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Externally-Expressed Fluorescence across Sexes, Life Stages, and Species of Spiders

by

Erin Brandt

A thesis submitted in partial fulfillment of the  
requirements for the degree of

Master of Science  
in  
Biology

Thesis Committee:  
Susan Masta, Chair  
Suzanne Estes  
Erik Sánchez

Portland State University  
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## Abstract

Although all spiders possess fluorophores in their hemolymph, the expression of external fluorescence is much more restricted. The purpose of this study was to evaluate differences in externally-expressed fluorescence between sexes, life stages, and species of spiders. To approach this question, we developed novel instrumentation to capture fluorescence with photographs of our specimens. We paired these fluorescence measurements with spectrometer measurements to attempt to determine the role that fluorescence plays in the overall coloration in spiders.

The study was divided into four sections. First, we examined how fluorescence varies in sexes and life stages in *Misumena vatia*, an ambush predator that typically preys on insects when they are on flowers. We found that adult females possess brighter external fluorescence than males in all body areas that we measured. We also found that external fluorescence remains relatively similar through life stages in females, but darkens over the course of a male's life. It is likely that the differences between males and females relate to differences in feeding ecology. External fluorescence may contribute to a visual signal allowing females to visually blend in with flowers.

The second study involved a series of experiments to determine whether freezing spiders at a temperature of  $-80^{\circ}\text{C}$  affects their fluorescence intensity. In spiders considered "white thomisids", fluorescence intensity increased after freezing, whereas

fluorescence brightness in darker-pigmented spiders did not change to any similar extent. It seems likely that tissue trauma due to freezing is the cause of increased fluorescence intensity after freezing.

The third study examined fluorescence brightness across ages and life stages of *Araneus diadematus*, a spider which is exposed to the sun, but builds large webs in which to snare prey. We found that, unlike *M. vatia*, adult males are the more brightly fluorescent sex, with adult females and all immature life stages possessing significantly less bright external fluorescence. It is unclear why these differences exist, but differences in ecology between adult males and all other life stages could play a role. Additionally, dim fluorescence may contribute to subtle patterning and/or convey photoprotection benefits to immatures and adult females.

In the final study, we examined external fluorescence across the Thomisidae family. Because of a relatively large number of species with a small sample size, we divided them into “white” and “dark” thomisids based on taxonomy and what is known about ecology. The white thomisids tend to be prey on insects on the exposed surfaces of flowers, whereas dark thomisids more often reside in leaf litter and crevices. We found that white thomisids fluoresce more brightly than dark thomisids. There were no differences between the sexes in either group, however. The differences between white and dark Thomisids may be related to differences in feeding ecology, whereas

males and females of the same group tend to have similar ecological characteristics, and also possess similar levels of fluorescence brightness.

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## CHAPTER 1: Intraspecific Differences in Fluorescence in *Misumena vatia*

### INTRODUCTION

Many animals use visual signaling to communicate with conspecifics, predators, competitors, and prey. There are many ways to generate such a visual signal, with the most common being by reflectance. In reflectance, light is bounced off of an organism in a way that can be perceived by others. The process of reflection does not change the incident light; the light is preferentially absorbed and reflected back based on properties of the surface of the organism to give the surface its perceived color.

Fluorescence is a more complex but related phenomenon which involves the absorption of one wavelength and the emission of another wavelength of light. This process occurs when photons of light excite molecules (fluorophores) to a higher electronic state. After excitation, one-photon fluorophores emit light that is of a longer wavelength (lower energy) than the excitation photons. Natural fluorophores can be found in multiple species of animals, including corals (Johnsen 2011), mantis shrimp (Mazel et al. 2004), scorpions (Kloock, Kubli, and Reynolds 2010), and at least one species of parrot (Arnold, Owens, and Marshall 2002).

Although fluorophores are widespread in nature, the fluorescence that they produce is not necessarily useful for the organism possessing them. For example, many researchers agree that fluorescence found in scorpions is not expressed in scorpions' natural environment, as ultraviolet excitation wavelengths are not present at night,

when scorpions are active. Thus, it is unlikely that scorpion fluorescence is biologically relevant (Kloock, Kubli, and Reynolds 2010). However, fluorescence occurs in many diverse taxa, making it impossible to dismiss its biological utility entirely.

Spiders represent one likely case of fluorescence serving an important function. Andrews *et al.* (2007) found fluorescence expression in a diversity of spiders across many different families. They also discovered that all spiders surveyed possessed fluorophores in their hemolymph, although the ability to fluoresce in a way that is visible at the surface of the animal is much more restricted. Brightly fluorescent spiders appear to have evolved multiple times as their distribution is scattered across a phylogenetic tree of spider relatedness (Andrews, Reed, and Masta 2007). Such a pattern of distribution is compatible with selection driving the expression of fluorescence. Spiders also vary with regard to sun exposure (and thus excitation wavelengths), as well as feeding behavior both within and between species. Thus, we wanted to explore whether variation in fluorescence brightness in spiders coincided with variations in ecological characteristics to determine whether external fluorescence served some purpose for the spiders possessing it.

For this study, we wanted to focus on the variation in fluorescence between sexes and life stages of one species of spider, *Misumena vatia* (Clerck 1757). This species was chosen for several reasons. First, it was important to insure that any results we obtained with fluorescence imaging equipment would reflect fluorescence that would be generated in the species' natural habitat. *M. vatia* is regularly exposed to the

sun at all life stages, so it is likely fluorophores present at the surface of the animal would fluoresce in a natural environment.

Additionally, we were interested in whether sexual dimorphism in fluorescence corresponds to more readily-apparent dimorphism, such as in size and color. *M. vatia* is markedly sexually dimorphic, in both size and color. Finally, it was important that variation existed between sexes in regards to feeding and prey choice that might correspond to differences in fluorescence. Again, there is variation between sexes in regard to feeding.

The purpose of this study was to determine whether externally-expressed fluorescence follows the same pattern of sexual dimorphism as the more readily-visible dimorphism in color and size in *M. vatia*. Also, we wanted to evaluate the change in fluorescence over the lifetime of a spider, from immature (insufficiently developed to determine sex), to penultimate males and females (one molt prior to adult, and able to determine sex), to adult males and females (sexually mature). We then wished to determine whether any differences in fluorescence paralleled the aforementioned feeding ecologies, to determine whether the expression of external fluorescence is selective in nature.

## METHODS

### **Study Organism**

*Misumena vatia* is a sit-and-wait predator in the family Thomisidae. This species has a Holarctic distribution, and individuals of all ages and sexes are diurnal predators

that often sit on flowers exposed to the sun while hunting. However, there are substantial differences in feeding habits between life stages and sexes. Adult females tend to position themselves on exposed flowers, waiting for large hymenopteran pollinators on which to prey. Adult males, on the other hand, spend more time pursuing or waiting for females with which to mate. Males tend to consume smaller prey than females do (Chien and Morse 1998) and eat just enough to maintain their mass, whereas females can sometimes increase their abdominal mass by an order of magnitude during their adult stage (Morse 2007).

There is also substantial sexual dimorphism within *M. vatia*. Females are generally white over most of their body with some individuals possessing a lateral red stripe on each side of the abdomen. Males are 10 times smaller than recently molted virgin adult females, and up to 100 times smaller than gravid females (Morse 2007, 221). Males also have darker patterning (maroon to brown hues), especially on the first two legs.

### **Specimen Collection**

*M. vatia* specimens were collected during the spring, summer, and early fall of 2007-2011. See Table 1.1 for collection locations. All *M. vatia* appear to be capable of reversibly changing to a yellow color in response to the color of the substrate (Morse 2007). However, only white *M. vatia* specimens were collected for the present study. Spiders that were collected as immatures were reared to adulthood in the lab prior to analysis. These spiders were kept in 7-dram vials on a 12-12 light cycle at room

temperature and fed fruit flies twice per week. All specimens were frozen at -80°C in 1.5 mL microcentrifuge tubes and maintained at that temperature until imaging.

### **Fluorophore Extraction**

To aid in the design of instrumentation to measure external fluorescence, we characterized the spectral characteristics of fluorophores found in *M. vatia* hemolymph. We extracted fluorophores from the abdomens of a few of the adult males and females assayed for external fluorescence intensity for this study. Fluorophores were extracted following the protocol indicated in Andrews et al. 2007. Briefly, we ground entire abdomens in 95% ultrapure ethanol. The sample was centrifuged to pellet any solid material and the resulting supernatant was used for fluorometry analysis. All fluorometry was performed in the laboratory of Scott Reed at the University of Colorado Denver using a PTI Spectrofluorometer. We also performed a parallel fluorophore study with specimens that had never been thawed or photographed.

### **External Fluorescence Excitation Light Source Determination and Calibration**

The generation and calibration of excitation wavelengths was an important aspect of generating biologically-significant levels of external fluorescence. Fluorophores typically have discrete peaks of excitation and emission wavelengths which are unique to each fluorophore. Thus, we needed to precisely control the wavelength of light used to illuminate the specimen for determination and documentation of whether the spider fluoresced.



After extensive experimentation, we determined that using light-emitting diodes (LEDs) was the best method of delivering precise wavelengths of light to a specimen. This is because each LED produces a fairly narrow band of wavelengths, unlike broad-spectrum ultraviolet light sources such as xenon-arc lamps. The use of a narrow range of wavelengths for excitation eliminates the necessity for more elaborate systems of filters that block all but the desired wavelengths.

To guide us in the selection of the light sources, we used fluorometer data of spider fluorophores from a previous study (Andrews, Reed, and Masta 2007) and from the present study. With the fluorometer data as a guide, we determined which LEDs produced wavelengths closest to those known to cause peak excitation of spider fluorophores.

We previously determined that peak excitation of spider fluorophores occurred at an excitation wavelength of approximately 290 nm for one fluorophore, and 330 nm for another fluorophore. For this study, we focused on determining if the fluorophores that excite at 330 nm are present in the surface of spiders. This wavelength is likely present in the light from the sun that would reach a diurnal spider, so emission measured at this wavelength would be seen in nature. Also, our equipment was unable to capture wavelengths in the range that would be necessary for the lower excitation wavelength. We did not know *a priori* whether other wavelengths of light would excite other potential fluorophores present in spiders, therefore we used a range

of different wavelength lights when imaging each spider. We were somewhat constrained by the wavelengths produced by LED manufacturers when choosing which LEDs to use, but we ultimately decided on four LEDs to use for fluorescence excitation, with peaks at 340 nm, 365 nm, 375 nm, and 400 nm, respectively.

Unfortunately, 340 nm was the lowest wavelength LED that could be easily acquired, although an LED with a peak at 330 nm would have been ideal. Also, the 400 nm LED was at the very edge of the ultraviolet range and out of the range of fluorophore excitation, so a good deal of the signal given off by images taken with that LED was strictly reflectance. However, we still used this LED to make sure we captured the full range of potential excitation wavelengths for the spider fluorophores.

In calibrating exposure times for the photographs with different LEDs, the wavelengths of light we used for illumination of the spider in the lab were made equivalent to those the spider would receive in natural conditions. To do this, the absolute irradiance of each LED powered to the manufacturer's specifications was measured and placed the same distance from the spectrometer probe that a specimen would be from the LED. Then, a similar spectral reading (absolute irradiance) of the sun on a sunny day was taken in typical spider habitat during the spring of 2011 (Figure 1.1). This spectrum was used as a reference spectrum because most of the spiders that were analyzed were active primarily in bright sunlight. In each case, the LEDs were dimmer than the solar reading, for their given wavelengths.

Next, the absolute irradiance curve for each LED was determined. We then integrated under the part of the curve comprising the range of wavelengths for the LED to obtain photons per second. The same range of wavelengths was integrated on the spectral curve of the sun. Since both readings were given in photons/second, one was able to calculate how many seconds of LED exposure would equal one second of exposure to those wavelengths given off by the sun.

The LEDs were extremely dim compared to the sun. For example, in order to expose a specimen to 1 second of the sun's light with the 340 nm LED, one would have to expose the specimen to the LED for over 20 minutes. With such a long exposure time, we were concerned that stray light in the room, no matter how dim, might distort the signal. This was especially a concern because while the sensitive Hamamatsu camera is well-suited to low-light situations, noise could also be amplified with long exposure times. There were also the practical considerations of the specimen drying out and moving between exposures, or the images being completely washed out by bright fluorescence. We eventually settled on exposure times that equal 0.10 second of the sun's light. These exposure times for each LED used (Table 1.2) were used for all fluorescence quantification photographs.

### **Instrumentation Design**

After excitation of external fluorophores, the resulting emission wavelengths were captured in photographs from which we were able to determine fluorescence

intensity. Directing excitation and emission wavelengths to the proper locations necessitated the design of specialized equipment.

Spider fluorophores present in hemolymph are excited in the ultraviolet range of light, with emission in the ultraviolet to visible range (Andrews, Reed, and Masta 2007). This presents unique challenges when designing instrumentation to capture images of externally-expressed fluorescence. Ultraviolet light does not pass through most standard optics without some degree of absorption and emission of a longer wavelength. This makes fluorescence emanating from the study subject difficult to discern (Johnsen 2011). Thus, design of specialized optical equipment was needed to evaluate fluorescence.

Images were captured using a model MVX10 Olympus stereo microscope with a 1x objective. The microscope was connected to an ultraviolet sensitive Orca R2 camera (Hamamatsu). The microscope was outfitted with a custom-built filter adapter to direct light to and from the specimen. A series of interchangeable UV LEDs were used to illuminate the specimen. The power to the LEDs was adjusted with a variable power supply. A diagram of the instrumentation is shown in Figure 1.2.

An important component of the fluorescence instrumentation was the dichroic beam splitter affixed inside the filter holder apparatus. The beam splitter had a 420 nm cutoff and was positioned at a 45 degree angle to both the light source and the specimen. When the light from the UV LED source hit the filter, the ultraviolet wavelengths were directed down 90 degrees to the specimen. When the specimen

fluoresced, the emission wavelengths (above 420 nm) were allowed to pass through the beam splitter and up toward the camera. A second blocking filter was installed above the dichroic filter, and before the light reached the camera. This blocking filter only allowed wavelengths longer than 450 nm through to the camera, thus ensuring that none of the ultraviolet light from the LEDs could pass through to the camera. This filter could be removed, and we captured images with and without this filter engaged to evaluate the possibility of some of the emission wavelengths also being below 450 nm.

### **Fluorescence Photography**

Specimens were allowed to defrost for several minutes after removing them from the -80 °C freezer, to allow ice crystals to melt and the spider to become flexible. Spiders were then pinned into the same position in order to standardize their position and exposure to the light source. Spiders were pinned with minuten pins (without piercing the specimen) to a substrate consisting of closed cell foam covered by clean, lint-free black velvet. The specimen was also visually examined at this point under a dissecting microscope and anything unusual (missing appendages, damaged body parts) was noted.

All subsequent measurements were made in a dark room from which any extraneous light sources in the room (such as those found on computers, smoke detectors, etc.) were removed or darkened. Spectrometer readings were taken immediately after the animal was pinned into position. Next, the specimen was placed under the microscope, brought into focus, and photographed under white light. All

images were taken at 1.6x magnification of the objective, for a total magnification of 16x. After a white-light image was taken, an LED was then attached to the filter holder apparatus and plugged in. For each LED, one image was then captured with the 450 nm blocking filter in place, and one image without the filter. Next, the LED was interchanged with the next lower wavelength LED and the process repeated. LEDs were used in a sequence from longest to shortest wavelength, because shorter wavelengths elicit a stronger response from the fluorophores, and thus present a higher likelihood of bleaching the fluorophore.

Although we found no evidence of bleaching, we also standardized our measurements by always taking spectrometer measurements before the fluorescence photography. It was important that great care be taken to not move the specimen on the stage of the microscope between photographs, because subsequent analyses of the white light image were used as comparators for all of the fluorescence images.

After photographs were taken at all four wavelengths, the spider was repositioned to bring another body part into focus, until all body parts were photographed. For *M. vatia*, we photographed the dorsal aspect of the abdomen, cephalothorax, and the first two pairs of legs. The legs were separated from the animal before being photographed, and the femur was always used as the region for maximal focus of the image. Body parts were not imaged for individuals if they were damaged or missing. Following

completion of all imaging, the specimen was stored in the -80 °C freezer in 95% ultrapure ethanol for later fluorophore extraction.

### **Image Analysis**

We used the ImagePro 7 Plus® software package to extract the data contained in the images of fluorescing spiders. To determine the brightness of the fluorescence we used a measure termed “pixel intensity” to determine the brightness of each pixel. The UV sensitive Orca camera captured images in black and white, and these were captured as 12 bit images.

Black and white 12-bit images assigned a numerical value to each pixel ranging from 0 to 4095. A value of 0 meant that the pixel is completely black and a pixel with the value of 4095 was completely white.

The white light image was loaded first into ImagePro®. Next, the “area of interest” tool was used to trace around the areas of the image to analyze, hereafter termed “area of interest” or “AOI”. This tracing process was performed using the white light image, because if a given specimen did not fluoresce brightly, it would be too difficult to see and trace the necessary areas of the image in fluorescence photographs.

Three AOIs were drawn for each body part imaged. When all AOIs had been drawn and saved, all fluorescence images were loaded into ImagePro®, and a macro was run that automatically applied each AOI to each image in sequence. After an AOI was applied, the macro automatically performed measurements and calculations based on

the circled region of the photograph. These calculations were exported to a .txt file for later processing.

In order to be able to measure contrast in the images, a second set of measurements were taken. We used the histogram feature in ImagePro®, which determined how many pixels were in a given brightness class. These measurements were also taken automatically with the same script used for the intensity measurements.

### **Data Analysis**

We used two main types of measurements to analyze all fluorescence image data: pixel intensity, and contrast. First, the average pixel intensity for each body region was calculated for each individual.

Normality of the average pixel intensity data was assessed with the Shapiro-Wilk normality test. To assess variance in fluorescence between sexes and life stages, we used single-factor ANOVAs. We also used Tukey's HSD post-hoc to establish significant differences between groups. For non-normal data, we used a Kruskal-Wallis test followed by a pairwise Wilcoxon test.

We also examined histograms to evaluate contrast. Histograms that depict pixel intensity could be automatically generated with ImagePro®. However, because there were 4096 levels of pixel intensity, histograms that depict this many different intensities



were overly complex. Therefore, we further processed the histogram data to make it more intuitive.

The pixel intensity range was divided into ten categories of brightness, with each category representing an equal tenth of the possible spread of pixels. These ten categories were then presented as a histogram. The ten classes of pixel intensity were further divided into three categories (dim, medium, and bright). Then, we calculated the percentage of area in a given body part that is occupied by pixels in the brightest category. The same statistical tests were used for this data as in the average pixel intensity data.

### **Reflectance Measurements**

We took reflectance measurements of each spider that was analyzed for external fluorescence. This was done to help determine what role fluorescence plays in the overall visual signal displayed by the spiders. Reflectance measurements were taken using a USB4000 spectrometer with a DH-2000 light source (from Ocean Optics) connected to a Dell laptop computer running Windows XP and Ocean Optics' Spectrasuite™ software. We used an integration time of 7 ms, and held the probe 2 mm from the specimen, at an angle of 90 degrees relative to the frontal plane of the spider. We used these settings because they had been used previously to gather spectrometric data from spiders (Heiling et al. 2005) Ten readings were averaged together per measurement. Measurements were repeated four times for each specimen.

Whenever possible, reflectance measurements were taken from each spider that was photographed and assessed for fluorescence. However, some males and all of the immatures were very small, so there is the possibility of decreased accuracy of the reflectance measurements for those individuals.

The resulting reflectance data could be assessed for significant differences with further data transformation (see Endler 1990 for a complete description) . However, the level of derivation required for such an analysis would not suit the purpose here of providing a general comparison between overall visual signal and fluorescence. Thus, reflectance data are presented without assessment of significant differences.

## RESULTS

### **Fluorophores Present in *M. vatia***

Peak excitation of *M. vatia* fluorophores occurred at 330 nm excitation. The resulting emission peak was bimodal, with peak emission between 390 nm and 460 nm. The fluorophores of males and females had similar emission peaks (Figure 1.4).

### **Average Fluorescence Intensity**

Emission wavelengths were visible in emission wavelength photographs of spiders (Figures 1.4, 1.5). Abdomen and cephalothorax data were normally distributed (Shapiro-Wilk  $p > 0.086$ ). To compare brightness in average pixel intensity between sexes and life stages, one-way ANOVAs were performed. For the leg data, a non-parametric Kruskal-Wallis test was performed. Fluorescence intensity differed significantly between sexes and life stages (see Table 1.3 for ANOVA and Kruskal-Wallis

results). See Figures 1.6-1.8 for p values associated with post-hoc Tukey's HSD (abdomen and cephalothorax and abdomen) and pairwise Wilcoxon tests (leg).

In all comparisons, adult female *M. vatia* fluoresced more brightly than males (Figures 1.6 -1.8). Adult females were brighter than adult males for all body regions when excited with a 340 nm LED light, and no cut-off filter was used (Figures 1.6-1.8). The abdomens of penultimate females fluoresced more brightly than those of adult females (Figure 1.6). Immature and penultimate male abdomens were about the same average intensity, and both were also about the same fluorescence intensity as adult females.

Adult and penultimate female cephalothoraxes displayed similar levels of fluorescence (Figure 1.7). Immature spiders did not fluoresce as brightly as either adult or penultimate females, and displayed dimmer pixel intensities. Penultimate males fluoresced less brightly than immature spiders, but brighter than adult males.

The legs of adult and penultimate females were not significantly different in average fluorescence intensity (Figure 1.8). The legs of penultimate males were dimmer than adult females, but brighter than adult males.

All of the above comparisons are based upon measurements taken with a 340 nm light source, without a 450 nm cut-off filter. Comparisons were also made among images taken with the 365 nm, 375 nm, and 400 nm LEDs. These differences in pixel intensity for the spiders for each of these conditions are illustrated in Figures 1.9-1.11.

### **Fluorescence as Measured by Percent Area in Brightest Category**

Adult and penultimate females had a greater area of their bodies in the brighter pixel intensity categories than adult males (Figures 1.12-1.15). A simplified overall histogram for representative individuals in each age/sex class can be found in Figure 1.12. In examining specifically the brightest third of pixel intensities, the data were not normally distributed and were assessed for variance with a Kruskal-Wallis test (see Table 1.4). However, significant differences between the groups were unable to be established via pairwise Wilcoxon tests due to small sample size and multiple tied ranks. A greater proportion of an adult female's body fluoresced brightly than a male's body (Figures 1.13-1.15). For all body parts, adult males had a smaller percentage of area in the brightest pixel category than females.

For the abdomen, penultimate males, adult females, penultimate females, and immature spiders had the same percent area in the brightest category (Figure 1.13). The cephalothoraxes of the different age and sex classes of *Misumena* individuals tended to have similar levels of fluorescence (Figure 1.14). Adult females, penultimate females, and immatures had similar percentages of bright area. Penultimate males had a similar percent in the brightest category as immatures, but had less bright area than adult and penultimate females. The legs of adult and penultimate females had similar percentages in the brightest category (Figure 1.15). Penultimate males and females were also the same, but penultimate males had less bright area than adult females. Penultimate males had the same percent as adult males. The fluorescence of the legs

of immature spider was not measured, as they were often too small to remove without damage.

Spider fluorescence when excited at 365 nm, 375 nm, and 400 nm, exhibited the same general trends in brightness as it did when excited with 340 nm light (Figures 1.16-1.18), although these data were not analyzed for significance.

### **Reflectance**

Reflectance measurements were averaged across sexes and life stages (Figure 1.19). Although significance was not established, some trends could be seen. Adult female spiders on average had the highest overall reflectance of any of the age classes of spiders examined, whereas adult males were on average the least reflective (Figure 1.19). Averages of Immature spiders had similar levels of reflectance as penultimate females, and both were more reflective than penultimate male spiders.

### **DISCUSSION**

The overall goal of this project was to characterize differences in external fluorescence between sexes and life stages in *M. vatia*. In order to do this, we photographed spiders under excitation wavelengths and measured the resulting fluorescence intensity in the photographs.

First, it is striking that the peak excitation wavelengths of the hemolymph fluorophores (330 nm; see Figure 1.19) coincided with the excitation wavelengths that produced the brightest images (340 nm; see Figures 1.6-1.11). This strongly suggests that fluorophores found in the hemolymph are either the same fluorophores found in

the surface of the spider, or that the fluorophores are different, but share the same spectral characteristics.

It is also likely that the fluorophore responsible for external fluorescence is present in both male and female *M. vatia*, given the similar fluorophore peak profiles (Figure 1.19). However, despite similarities in the spectral properties of fluorophores between the sexes, external fluorescence intensity varies drastically between males and females, and through the life of a spider.

Average fluorescence intensity changed across some of the life stages of *M. vatia*. The abdomens of immature *M. vatia* fluoresced brightly, but at the penultimate stage, males and females began to differ. Penultimate females continued to fluoresce more brightly as they matured, while penultimate males did not differ from the immatures. At the adult stage, males and females were markedly different, with adult females much brighter than the males. There was a similar pattern in the cephalothorax. However, males and females began to differ at the penultimate stage. In the front legs, brightness did not change at any time during the life of the female, but in males, the darkening began at the penultimate stage. This darkening continued into the adult stage.

Since the spectral properties of the fluorophores in adult males and females are similar, this suggests that the fluorophores of both sexes possess the same physiological properties and constraints. Therefore, the lack of sexual dimorphism in fluorophores, but the presence of sexual dimorphism in the expression of fluorescence suggests that

natural selection acts differently on external fluorescence expression in males than females, and through life stages.

However, before discussing the ways in which selection has shaped external fluorescence in *M. vatia*, we must first discuss whether such fluorescence contributed to the overall visual signal that the spiders presented.

### **Fluorescence as a Visual Signal**

An ultimate goal of this study was to determine how fluorescence impacts the overall visual signal, which consists of both reflectance and fluorescence. Approaching this question can be technically difficult. During reflectance measurements, fluorophores are also excited. Thus, reflectance measurements were actually representative of reflectance and fluorescence combined. It is possible to mask a specimen at the excitation wavelengths so that fluorescence is suppressed and only reflectance is observed (Fuchs 2001). However, it is then impossible to determine the level of reflectance at those excitation wavelengths due to non-fluorophore pigments. In *M. vatia*, the excitation wavelengths are in the ultraviolet. Since UV reflectance is important to the visual systems of *M. vatia*'s prey (Heiling et al. 2005), masking those wavelengths to stop fluorescence would also remove a vital portion of the reflectance spectrum.

To work around this problem, we used a dual approach whereby we took both spectrometer measurements and photographs to assess the overall visual signal and fluorescence, respectively. The problem with using two separate methods is that the

units are not directly comparable. In the case of *M. vatia* we compared the relative reflectance of different sexes and life stages at the known emission peaks of the fluorophores with relative fluorescence intensity from photographs.

In adult females, there is both bright reflectance and fluorescence, indicating that fluorescence probably does play an important role to the overall visual signal, perhaps reinforcing a reflectance signal (Figures 1.3, 1.19). This pattern holds true for the adult males, which are both the dimmest in fluorescence and reflectance (Figures 1.3, 1.19). Thus, we might surmise that fluorescence in adult males does not play an important role in enhancing a visual signal. Interestingly, penultimate females and immatures have similar levels of reflectance at emission wavelengths, but immatures fluoresce less brightly. This suggests that even though penultimate females fluoresce brightly, the overall signal is less affected by fluorescence than it is for the adult females.

The differences that we see in fluorescence intensity vary widely and can be predicted based upon the sex and developmental stage of the spider. This implies that there is some selective pressure either to repress fluorescence in males, or to exaggerate it in females and juveniles. Below we outline several possible ways that selection may be acting to make fluorescence brighter in adult females, penultimate females, and immatures, and dimmer in adult and penultimate males.



### **No selective function of fluorescence**

One possibility for the presence of externally-expressed fluorescence in *M. vatia* is that it serves no function, and is simply an incidental effect of a chemical process occurring as part of the spider's metabolism. If this was the case, we may expect to see that all age classes and sexes of spiders exhibit the same fluorescence expression.

However, our fluorescence data do not support the hypothesis that fluorescence serves no function. We do not see a random distribution of fluorescence intensity within and between life stages and sexes. Additionally, if fluorescence intensity was randomly distributed among individuals, we would not be able to predict the intensity of an individual based upon its sex or life stage. Instead, we find just the opposite with striking differences in fluorescence expression between adult males and females.

### **Photoprotective (Sunscreen Effect)**

Because high-energy wavelengths are emitted by the sun, and some of those wavelengths arrive unattenuated at the surface of the earth, there is the risk for organisms exposed to the sun to experience damage. Especially dangerous are ultraviolet wavelengths, which can cause tissue damage, DNA damage, and cancer (Gallagher and Lee 2006). Fluorophores, by their very nature, change high-energy wavelengths to low-energy wavelengths (Johnsen 2011). Thus, we might expect that fluorophores function as a sort of sunscreen to protect organisms from damaging ultraviolet radiation. This hypothesis has been proposed for fluorescent corals which are often exposed to the sun at low tides (Reef, Kaniewska, and Hoegh-Guldberg 2009;

Salih et al. 2000). *M. vatia* fluorophores specifically convert ultraviolet light to visible light, so it is possible that fluorescence may serve a photoprotective function.

If spider fluorophores' primary function was photoprotective in nature, we would first expect the excitation wavelengths of the fluorophores to be dangerous, high-energy wavelengths. We would also expect to see fluorescence intensity directly proportional to sun exposure. Finally, the individuals with the greatest need of avoiding DNA damage (adult females containing developing embryos) should possess the brightest fluorescence.

The fluorescence intensity data do not completely agree with these necessary preconditions. First, wavelengths at the peak excitation for the fluorophore found in *M. vatia* (330 nm) (Figures 1.3, 1.4) are within the near ultraviolet range (UVA), which are less damaging to tissue than lower ultraviolet wavelengths (Gallagher and Lee 2006, but see Kligman, Akin, and Kligman 1985; Cole 2001). It seems unlikely that photoprotective fluorophores would be necessary to deal with these less-dangerous wavelengths. Additionally, we do not see fluorescence intensity as directly proportional to sun exposure. Adult males are more mobile and seem to eat less, but still do their hunting on flowers exposed to the sun (Morse 2007).

Finally, penultimate females fluoresce more brightly than adult females. It seems counterintuitive that adult females would require less ultraviolet protection. Adult female abdomens often become extremely distended with eggs (Morse 2007). Egg production requires that the cuticle stretch and presumably become thinner and

more transparent to light, presumably including dangerous ultraviolet wavelengths. It seems, then, that adult females (and the embryos contained with them) would benefit from increased, not decreased, fluorescence if its function is to protect from ultraviolet light.

It seems unlikely that the function of fluorescence in *M. vatia* caused by the fluorophore of interest primarily serves a photoprotective function. However, there is the possibility that the fluorophore that was excited by 340 nm wavelengths does provide an ultraviolet-protective role, albeit a minor one in addition to other functions. We also cannot dismiss the possibility that another fluorophore with lower excitation and emission wavelengths does perform an ultraviolet-protective function.

### **Prey attraction**

Another possibility for the purpose of fluorescence in spiders could be to accentuate a visual signal that prey organisms find attractive. Several studies have found evidence that in some spiders, body coloration and pattern serves to attract prey (Bush, Yu, and Herberstein 2008; Herberstein, Heiling, and Cheng 2009).

If this were the case, we would expect that prey species are drawn to flowers with spiders sitting on them. This does not seem to be the case, at least with *M. vatia* studied in the eastern United States. Generally, hymenopteran prey tend to avoid flowers with crab spiders on them, with the exception of large bees, which presumably are at lesser risk of being eaten due to their large relative size (Dukas and Morse 2003). Interestingly, some dipterans seem to be attracted to emission wavelengths that we find

to be present in the *M. vatia* fluorophores (Diclaro et al. 2012). However, there are indications that syrphid flies specifically, which are the main food source for immatures and males, are more attracted to longer (520 nm - 600 nm wavelengths) (Laubertie, Wratten, and Sedcole 2006; Wacht, Lunau, and Hansen 1996). There are no experimental data to suggest that flies are specifically drawn to flowers with spiders.

### **Camouflage**

An ecological hypothesis for the function of fluorescence in *M. vatia* relates to camouflage. If bright fluorescence is used for camouflage, then we would expect to see bright fluorescence in individuals that a) hunt visually-oriented prey that may be fooled by camouflage and/or b) individuals that need to hide from visually-acute predators. Dimmer fluorescence would be seen in individuals that do not need to attract visually-acute prey and/or do not need to hide from predators.

Adult female *M. vatia* are often well-blended into their surroundings, possibly to avoid being detected by prey (Thery et al. 2005; Morse 2007). Bright fluorescence could work to enhance this visual signal, allowing spiders to blend in with the flower on which they wait for prey. How do we explain, then, the comparatively dimmer fluorescence in males? One way to answer this question is to look at feeding ecology and risk avoidance.

Large and potentially dangerous hymenoptera seem to rely primarily on color for distinguishing a predator from the background, and have difficulty associating the shape of a camouflaged spider with danger, given no prior experience (Ings, Wang, and Chittka

2012). By being less fluorescent, and thus dimmer and more likely to contrast with a flower, perhaps the bees would be more able to see the males, and thus avoid landing on a male-occupied flower.

Interestingly, males are not uniformly dim; their legs are by far the darkest part of their bodies (figures 1.4 – 1.8). Perhaps the dark legs specifically project a “spider-like” shape to hymenopterans, or perhaps it is physiologically more difficult to suppress fluorescence on the abdomen or cephalothorax. If hymenopterans are discouraged from landing on a flower by visually ascertaining the presence of a male, this could then prevent a potentially dangerous confrontation for the male spider. Males keeping large bees away from visiting flowers could potentially chase valuable food resources away from the female that the male is courting. However, according to Holdsworth and Morse, the mating process only lasts about 4 minutes, and males rarely guard females (Holdsworth and Morse 2000), although we have personally observed mate guarding in the field. Males may not be with any given female long enough to drive away sufficient prey to negatively impact her fitness, even if they are engaging in some form of mate-guarding.

Conversely, dipterans, especially syrphid flies (species which males are more likely to eat), seem not to notice spiders on flowers as readily as hymenopterans (Brechtbuhl, Casas, and Bacher 2009; Romero, Antiqueira, and Koricheva 2011). Perhaps the slight change in visual signal by the males is sufficient to keep dangerous hymenopterans away, while still maintaining sufficient camouflage to insure that

enough flies visit the flower to sustain the male. Adult female *M. vatia* have also been shown to indiscriminately feed on syrphid flies (Morse 2007), so this could prevent the female from leaving the flower with the male to find better feeding grounds.

Curiously, we also see that immature *M. vatia* are brightly fluorescent. However, they are also too small to consume a large hymenopteran prey (Morse 2007, p. 59) and could also be at risk from such a large animal like the males. Why, then, are the immature instars brightly fluorescent, and not dim like the adult males? To answer this question, we can look to predation risk avoidance.

Immature *M. vatia* are at great risk for predation, primarily by jumping spiders (Morse 1992). In this case, immature *M. vatia* instars would need good camouflage to hide from keenly visual salticid predators (Peaslee and Wilson 1989; Harland and Jackson 2000). Although good camouflage could still put small immature spiders at risk from a dangerous confrontation with a large hymenopteran, the risk of predation from other spiders could make an occasional encounter with a bee negligible in terms of selection.

Adult males, on the other hand, are highly mobile due to their long legs, and have extremely low rates of predation (Morse 2007, 213). This suggests that even if males are preferentially targeted by certain predators due to their lack of camouflage, they could easily get away to avoid predation.

## CAVEATS

Several important caveats need to be considered when examining *M. vatia* ecology. First, to our knowledge, there have been no studies of the ecology of *M. vatia* from the region our specimens were collected. Predator, prey, and substrate plant species are different in the Pacific Northwest region of the United States than where ecological studies have been conducted. Importantly, Morse has noted that he has never documented a predation event on *M. vatia* by a bird (Morse 2007, 24). However, there is a different suite of bird species in the Pacific Northwest. As birds are highly visual predators (Hill and McGraw 2006), any signal that *M. vatia* gives off via fluorescence could be affected by this possible predation pressure.

In addition, although some studies have suggested that *Misumena* coloration is selected to provide camouflage from prey species (Brechtbuhl, Casas, and Bacher 2009; Ings, Wang, and Chittka 2012), we did not perform similar substrate-matching experiments with our spiders, partially due to the difficulty of performing spectrometer measurements on the complex preferred host flowers. There have been no documented regional differences in coloration described so far, however closely related species of Thomisid spiders, with seemingly similar coloration have been shown to be quite different, with different effects on prey species (Thery and Casas 2002; Heiling et al. 2005; Herberstein, Heiling, and Cheng 2009; Llandres et al. 2011).

As a final caveat, we have put forward several hypotheses regarding *M. vatia* camouflage in the context of hymenopteran visual systems. Hymenoptera is an extremely diverse group, and the visual systems of only a few well-studied species are

known. It is entirely possible that *M. vatia* prey on species with visual systems unlike the large hymenopteran species that happen to have well-studied visual systems.

In addition to the fluorescence-quantification work that we performed, important ecological experimentation should be conducted. First, some of the ecological data, including feeding ecology, intrasexual interactions, and predator dynamics should be explored for *M. vatia* in the Pacific Northwest similar to the work done by Morse in the Eastern United States. Also manipulative experimentation should be conducted to help elucidate possible ecological impacts of fluorescence, possibly examining fluorescence and how manipulating it affects individuals at various life stages.



## TABLES AND FIGURES

**Table 1.1** Collection counties for *M. vatia* specimens

<b>Oregon</b>	
	Clackamas
	Coos
	Douglas
	Hood River
	Josephine
	Klickitat
	Lincoln
	Linn
	Multnomah
	Sherman
	Wasco
<b>Washington</b>	
	Skamania

**Table 1.2** Exposure times used for fluorescence photography

Exposure times were calibrated such that the number of photons given off would equal the same number of photons given off by the sun at that wavelength in .10 seconds

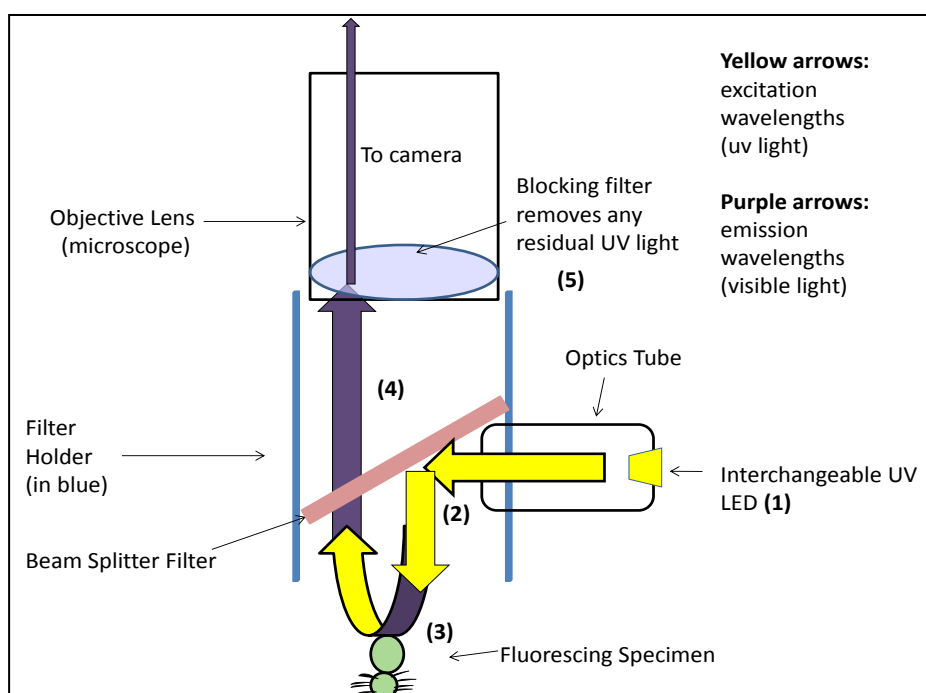
LED used	340 nm	365 nm	375 nm	400 nm
Exposure time (s)	117.05	38.88	2.27	0.98

**Table 1.3.** ANOVA/Kruskal-Wallis results for average intensity of all body parts. The asterisk indicates a p value generated by a Kruskal-Wallis test.

	F	F <sub>crit</sub>	p
abdomen	8.94	2.68	$6.40 \times 10^{-5}$
cephalothorax	12.82	2.68	$2.87 \times 10^{-6}$
right leg 1	N/A	N/A	$8.36 \times 10^{-4*}$

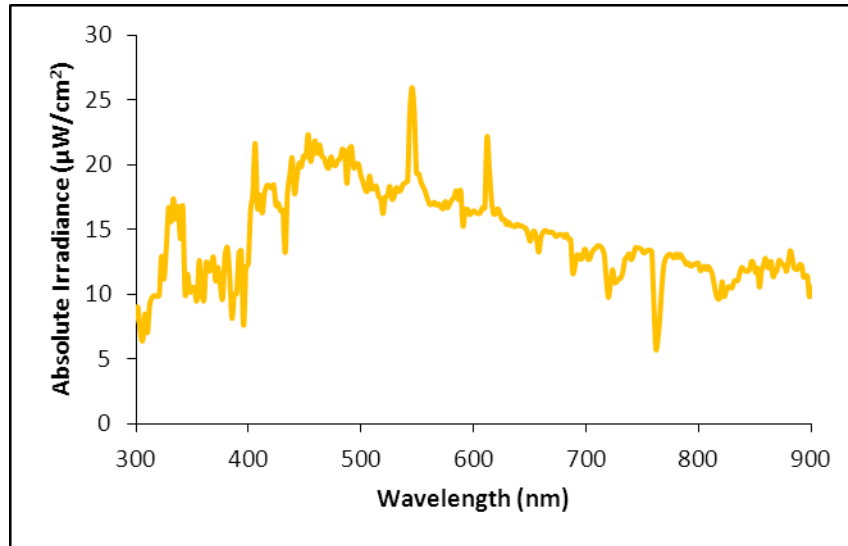
**Table 1.4.** Kruskal-Wallis results for histogram of all body parts.

	p
abdomen	$3.34 \times 10^{-2}$
cephalothorax	$3.2 \times 10^{-4}$
right leg 1	$3.67 \times 10^{-2}$

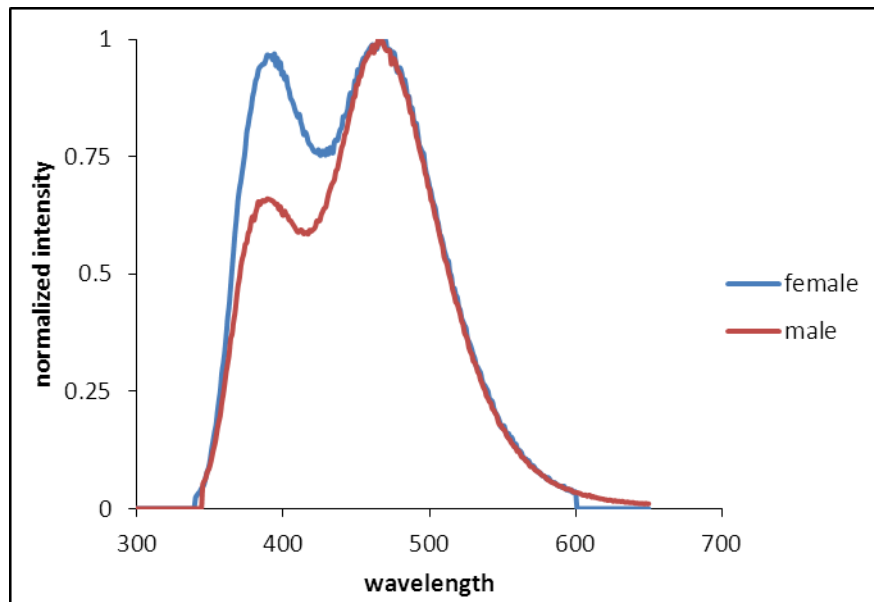


**Figure 1.1.** Fluorescence Photography Instrumentation

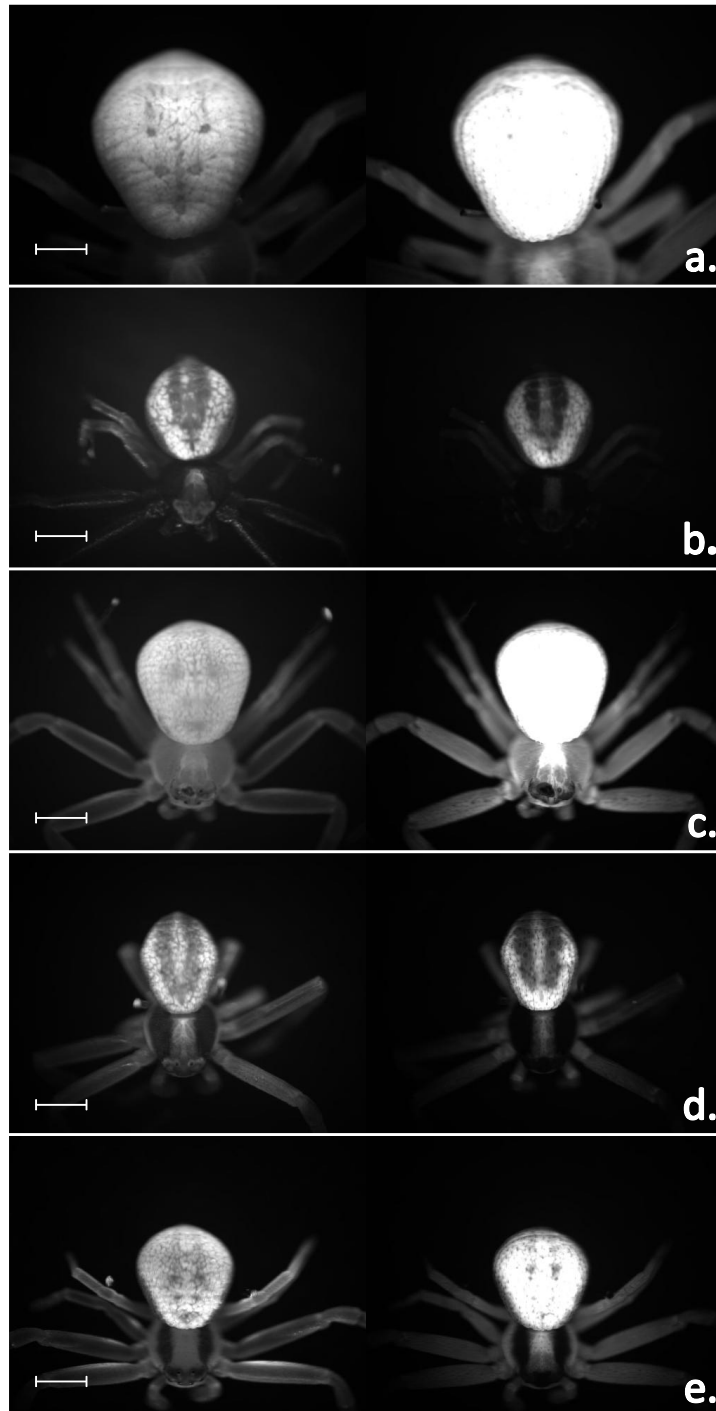
- 1) Light is produced by ultraviolet LED and passes through the optics tube
- 2) UV light (below 425 nm) light is directed downward by a beam splitter (dichroic) filter and down to the specimen
- 3) Fluorophores within specimen convert ultraviolet wavelengths to visible light
- 4) Visible wavelengths pass up through the beam splitter
- 5) A blocking filter blocks any light below 450 nm and the remaining light goes up through the microscope to be captured by the camera



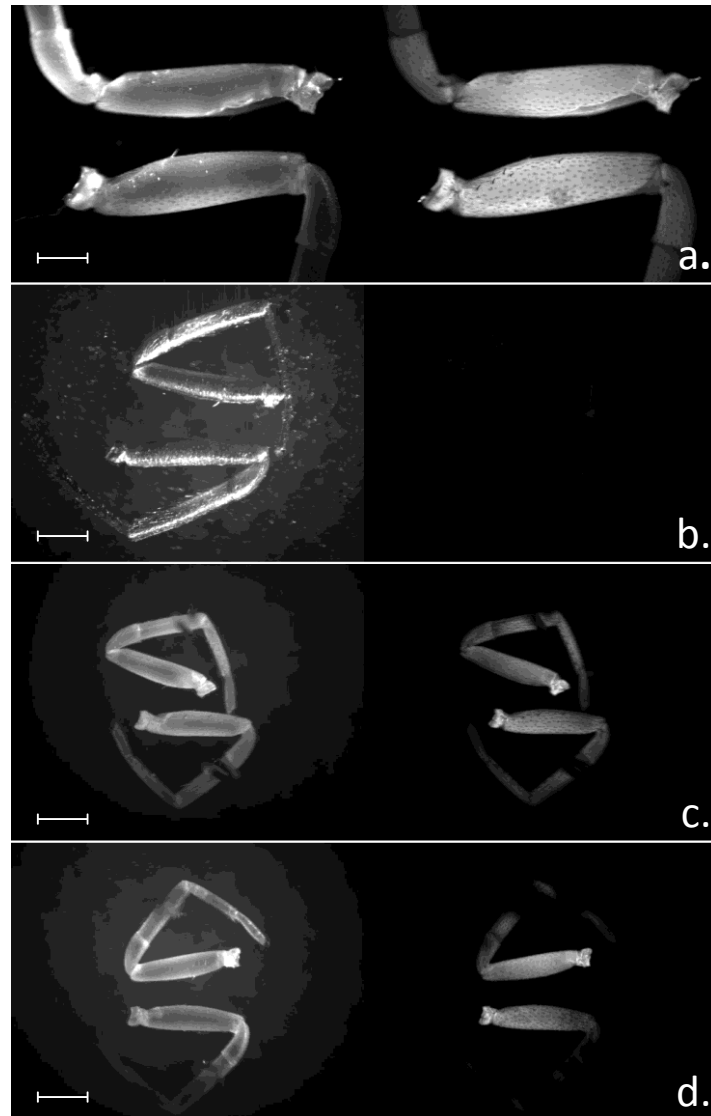
**Figure 1.2.** Absolute Irradiance of the Sun at Sea Level.  
Readings were taken on PSU campus 4-27-2011 at 3:31 pm with a USB4000 Spectrometer



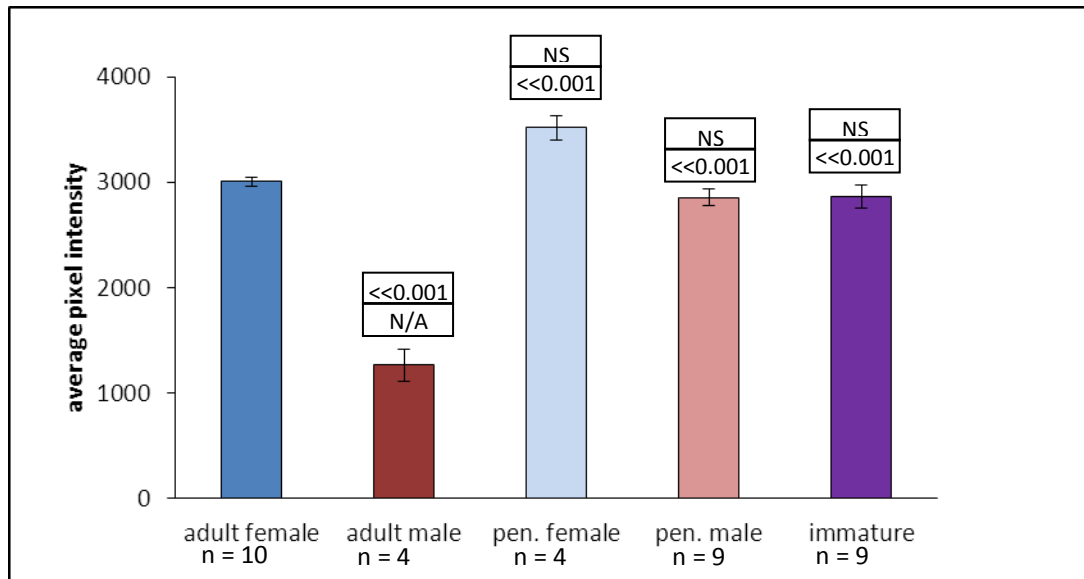
**Figure 1.3.** Normalized emission of fluorophores from representative adult male and adult female *M. vatia* individuals excited with 330 nm light. These individuals were not previously imaged or thawed.



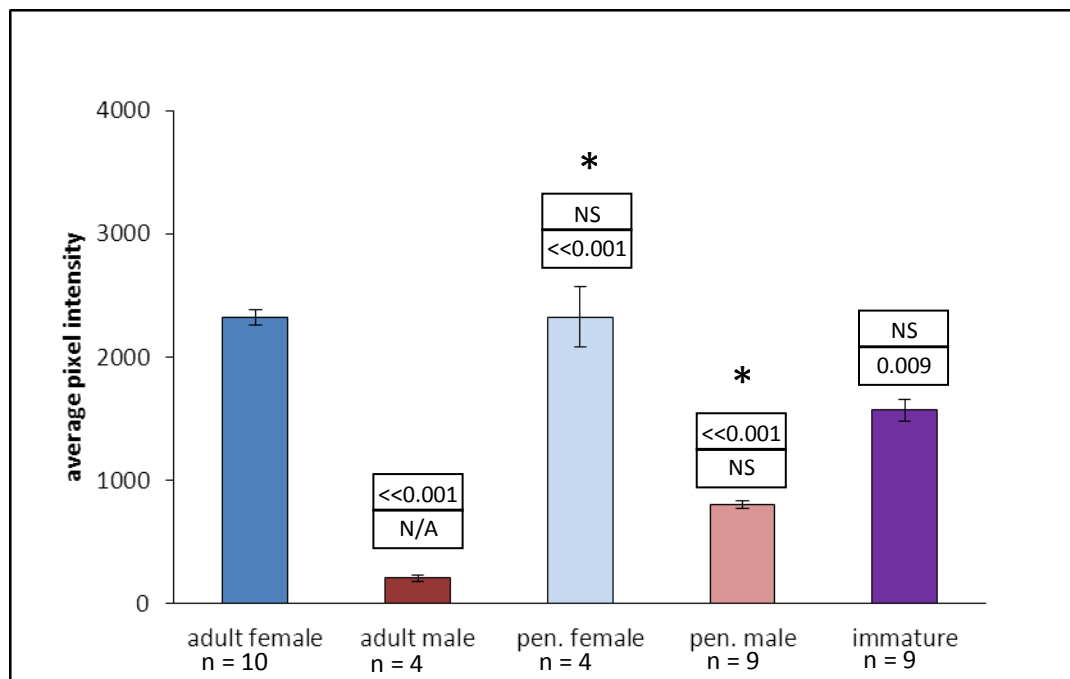
**Figure 1.4.** Representative images of *M. vatia* abdomens under UV light. Left panel represents white light image, and left panel represents 340 nm exposure without blocking filter for (a) adult female, (b) adult male, (c) penultimate female, (d) penultimate male and (e) immature specimens. Bar represents 1 mm.



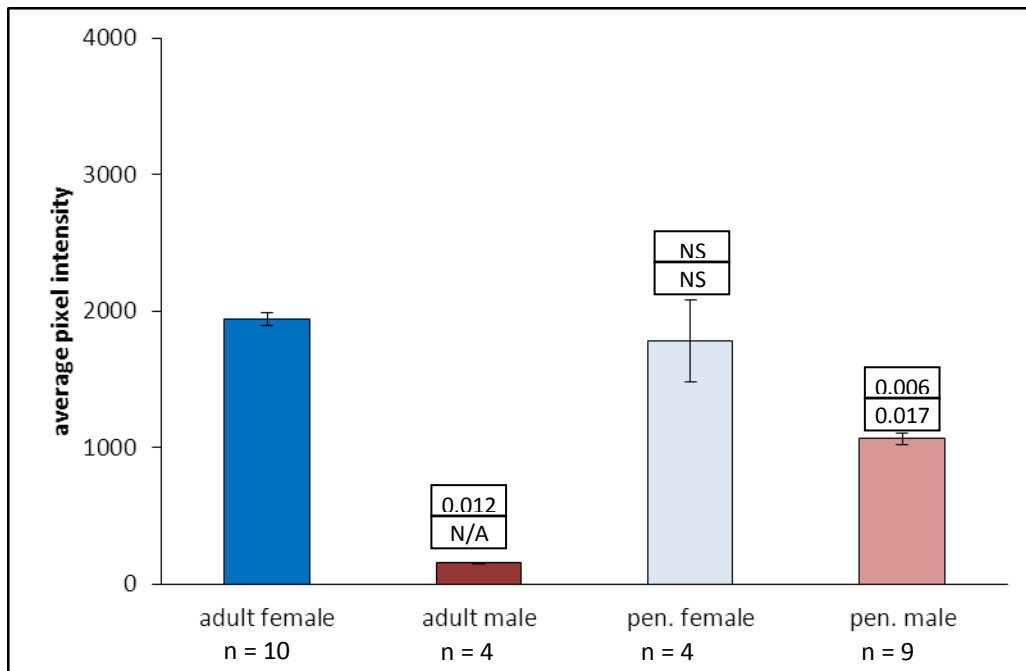
**Figure 1.5.** Representative images of *M. vatia* legs under UV light. Left panel represents white light image, and right panel represents 340 nm exposure without blocking filter for (a) adult female, (b) adult male, (c) penultimate female, (d) penultimate male specimens. Bar = 1 mm.



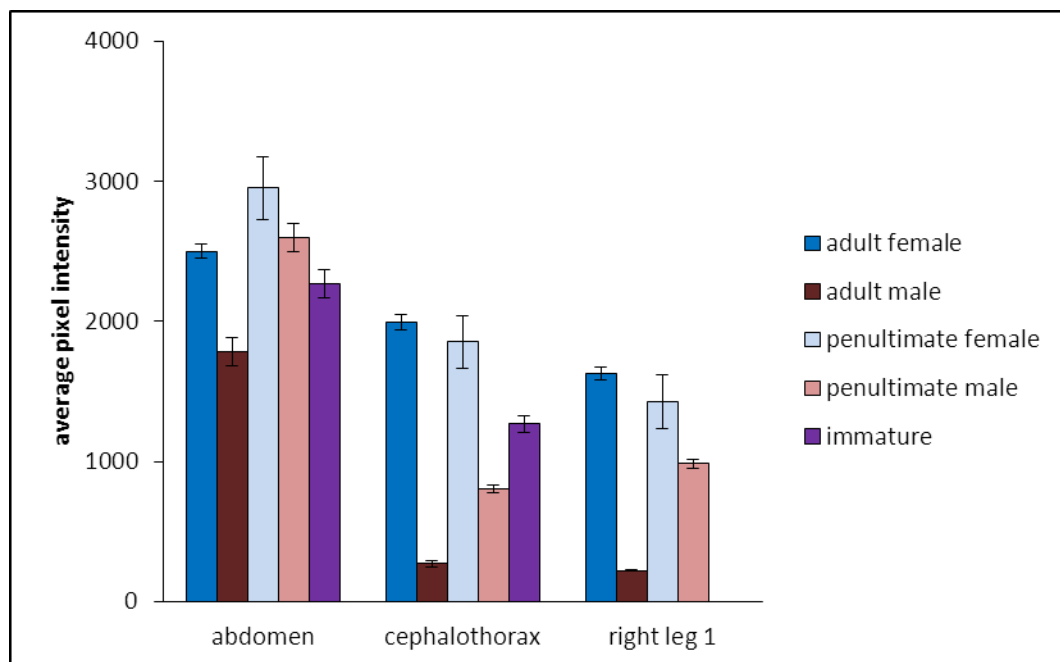
**Figure 1.6.** Average fluorescence intensity at 340 nm without the blocking filter for the abdomens of *M. vatia*. Intensity was calculated on a pixel-by-pixel basis. P values for differences between each group and adult female (top box) and adult male (bottom box) are shown (Tukey's HSD).



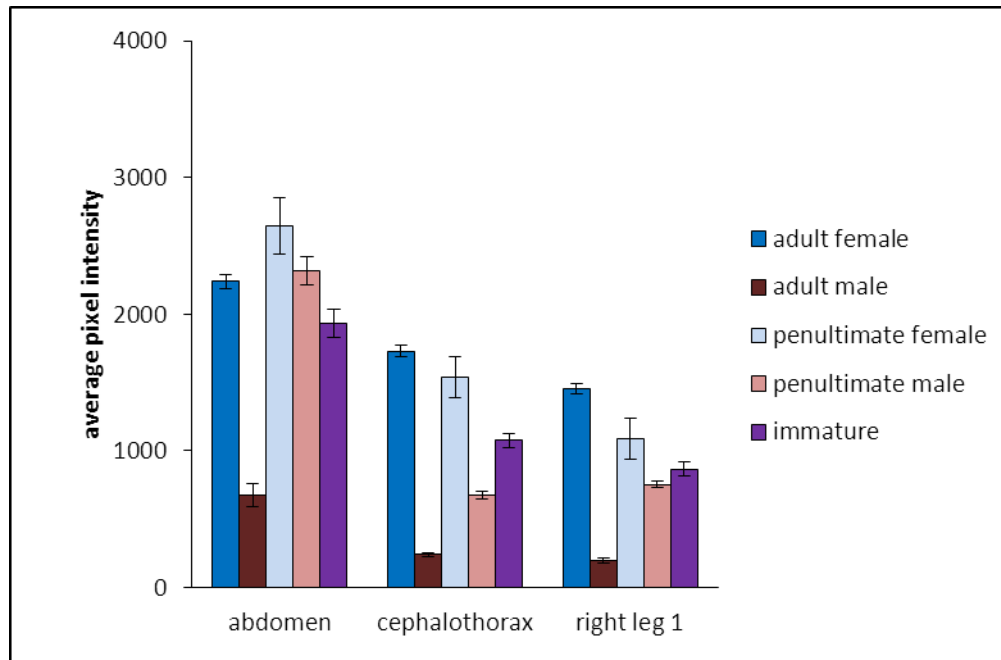
**Figure 1.7.** Average fluorescence intensity at 340 nm without the blocking filter for the cephalothoraxes of *M. vatia*. Intensity was calculated on a pixel-by-pixel basis. P values for differences between each group and adult female (top box) and adult male (bottom box) are shown. Groups marked with \* differ with a p value of 0.003 (Tukey's HSD).



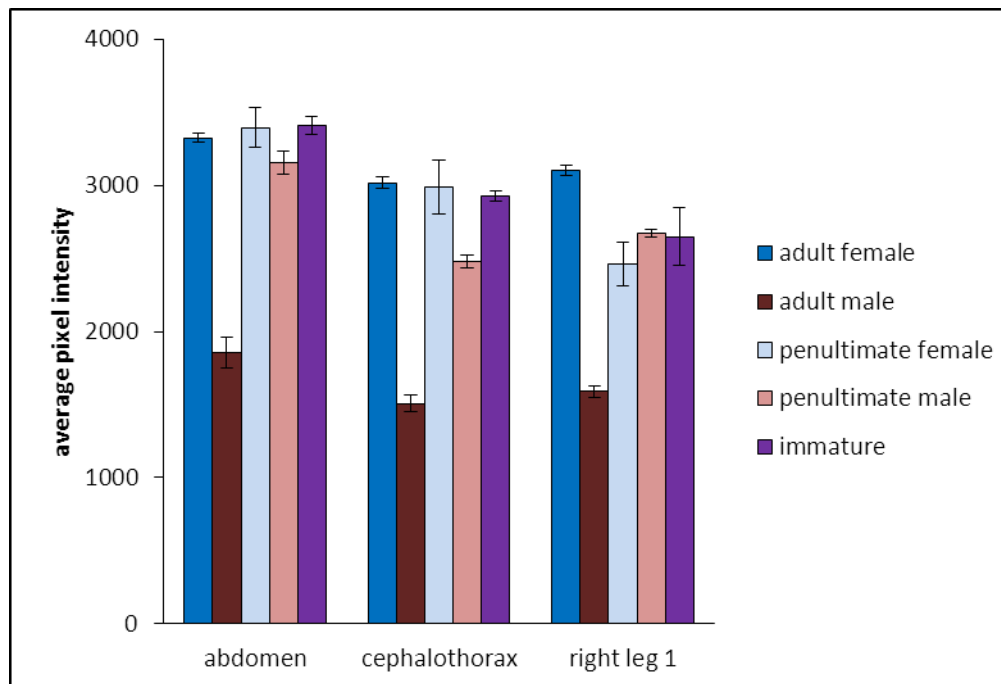
**Figure 1.8.** Average fluorescence intensity at 340 nm without the blocking filter for the right leg 1 of *M. vatia*. Intensity was calculated on a pixel-by-pixel basis. P values for differences between each group and adult female (top box) and adult male (bottom box) are shown (Pairwise Wilcoxon Test).



**Figure 1.9.** Average fluorescence intensity at 365 nm in *M. vatia* without blocking filter. Intensity was calculated on a pixel-by-pixel basis for each body part indicated

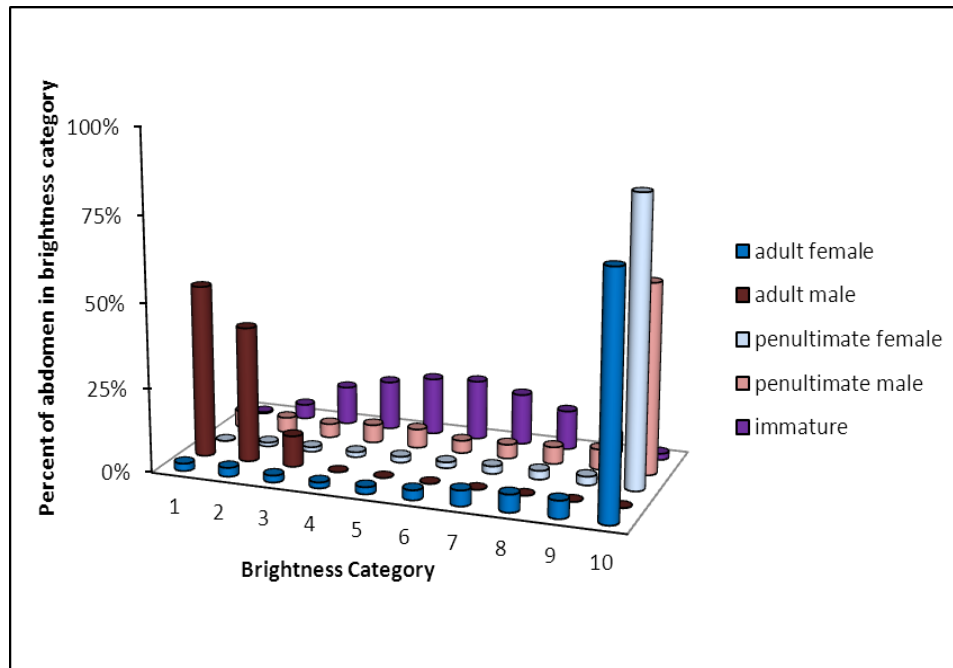


**Figure 1.10.** Average fluorescence intensity in *M. vatia* at 375 nm without blocking filter. Intensity was calculated on a pixel-by-pixel basis for each body part indicated

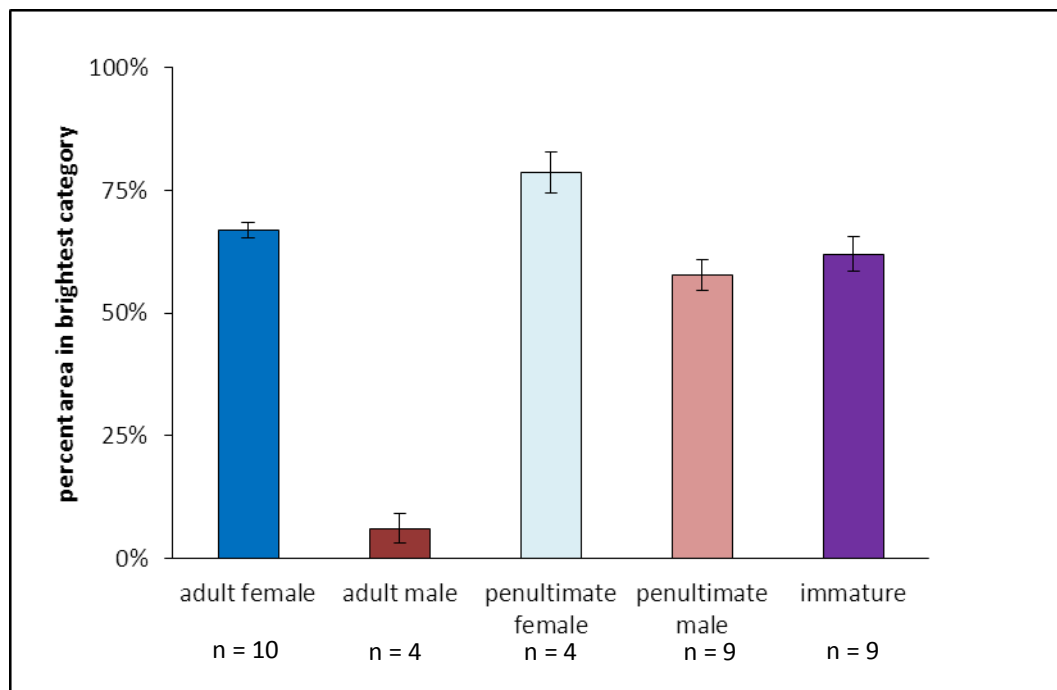


**Figure 1.11.** Average fluorescence intensity in *M. vatia* at 400 nm without blocking filter. Intensity was calculated on a pixel-by-pixel basis for each body part indicated

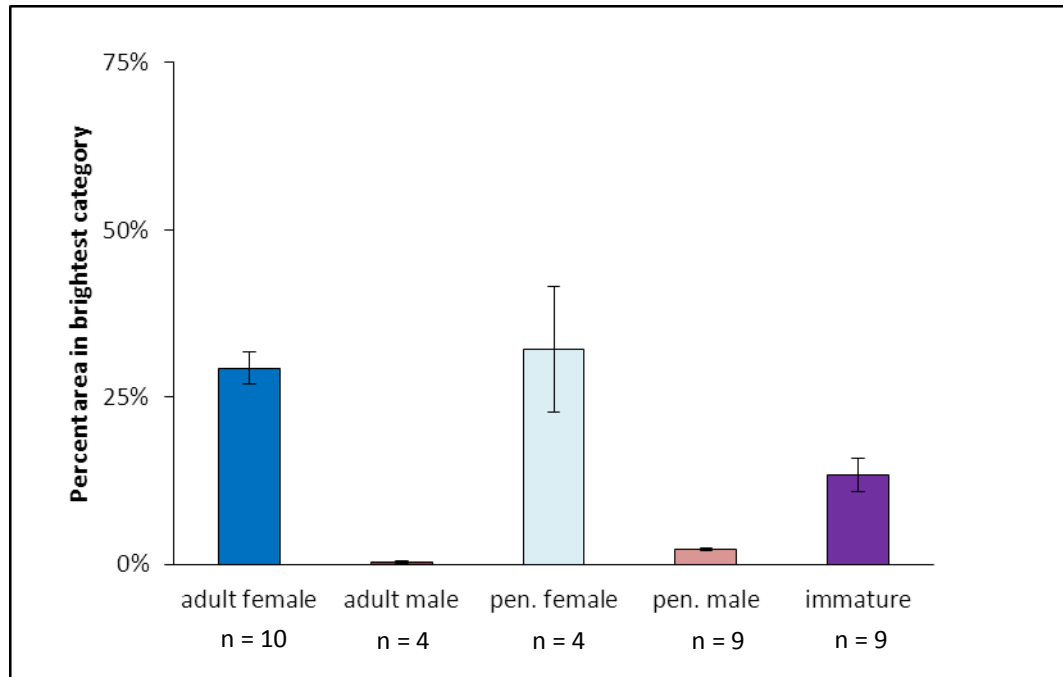




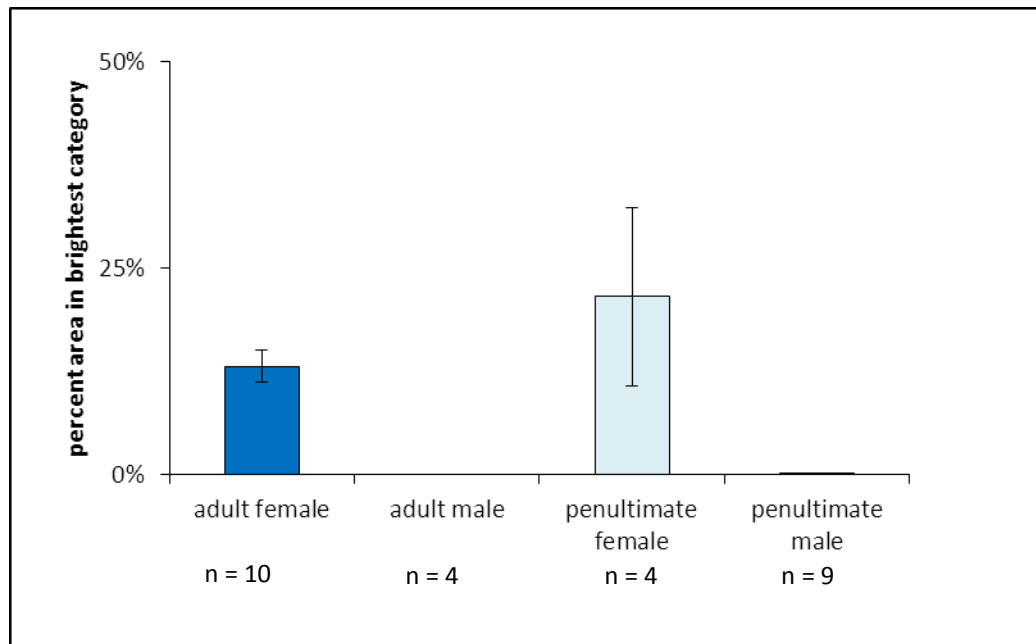
**Figure 1.12.** Simplified histogram of fluorescence in abdomens of representative *M. vatia* specimens at different life stages. Histogram has been broken into 10 categories of brightness for clarity.



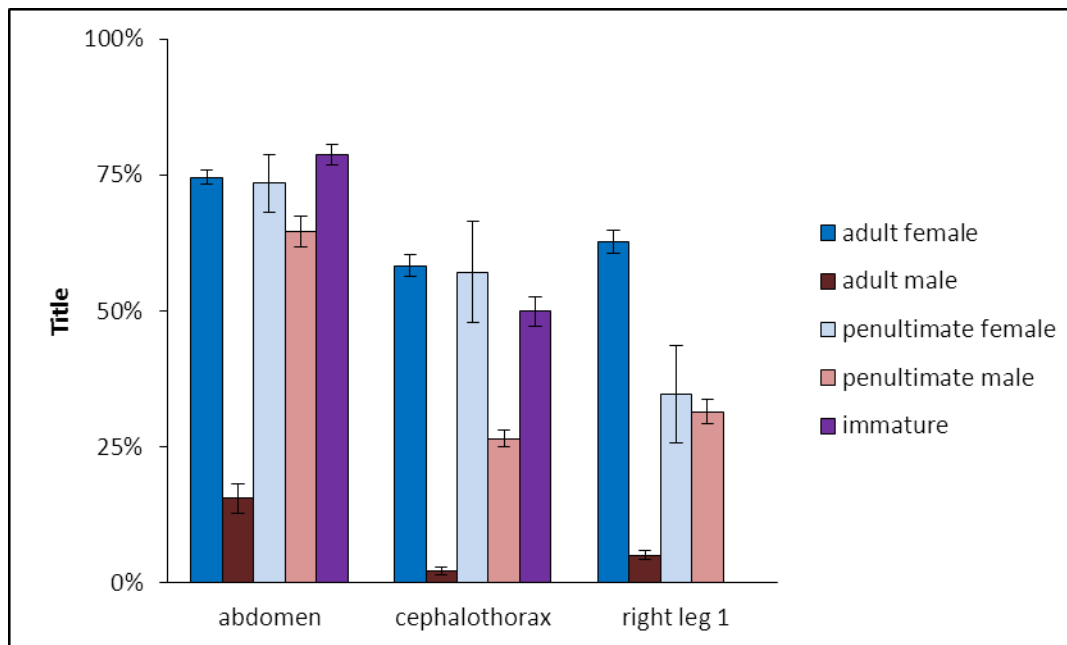
**Figure 1.13.** Percent area of abdomen in *M. vatia* in brightest category at 340 nm without blocking filter. Brightness category is defined as the highest third of possible pixel intensity values. Numbers above bars indicate p value for difference from adult male (Tukey's HSD).



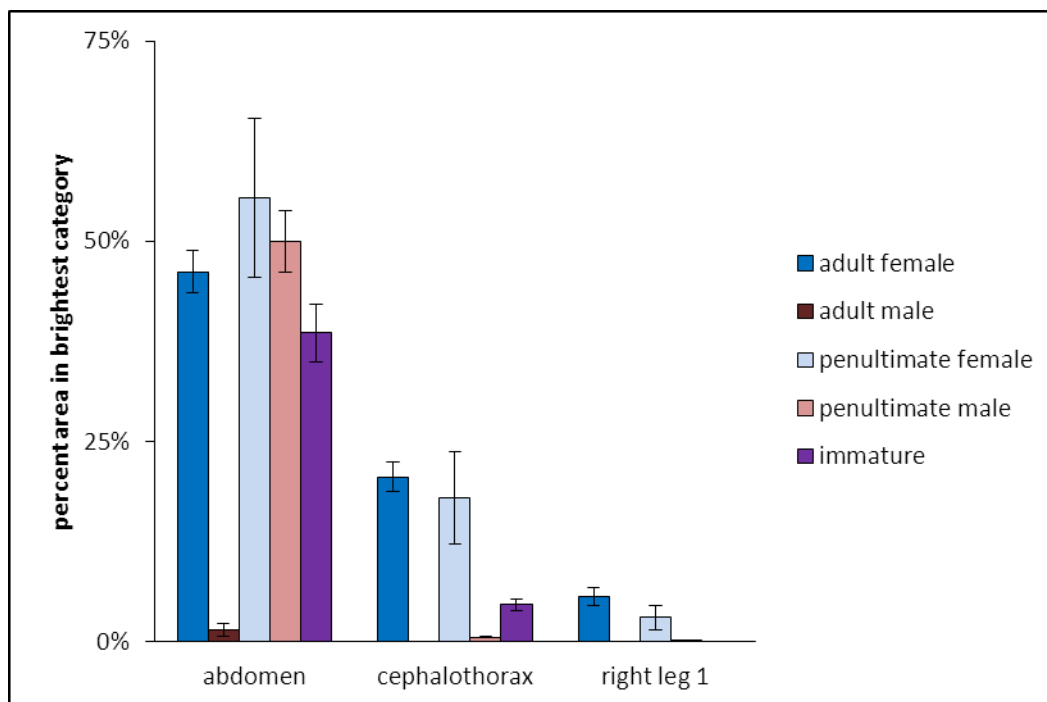
**Figure 1.14.** Percent area of cephalothoraxes in *M. vatia* in brightest category at 340 nm without blocking filter. Brightness category is defined as highest third of possible pixel intensity values.



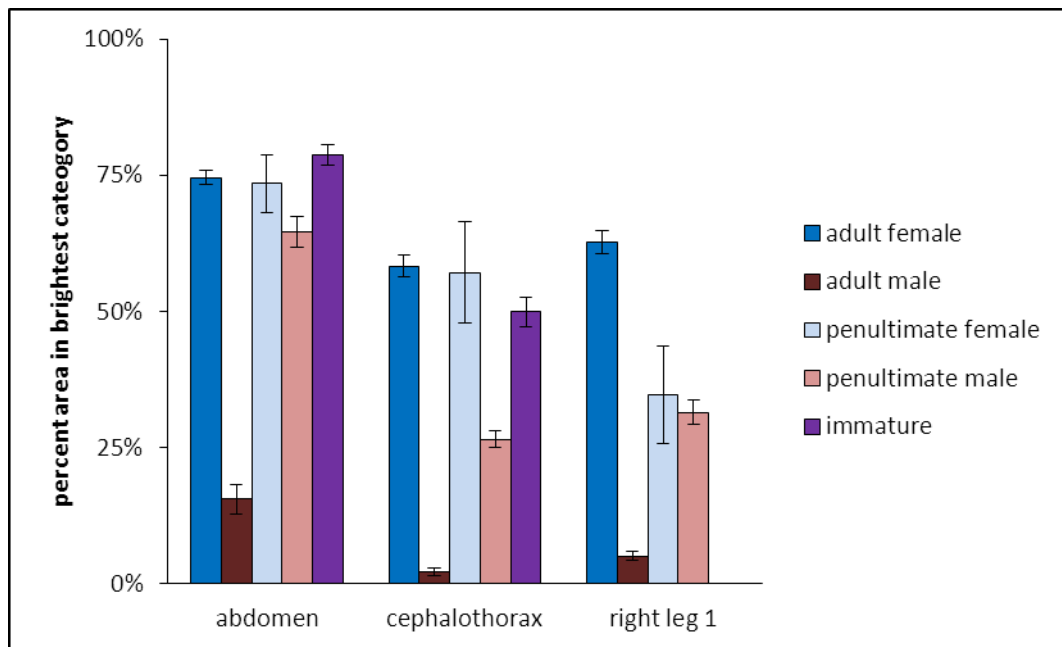
**Figure 1.15.** Percent area of right leg 1 in *M. vatia* in brightest category at 365 nm without blocking filter. Brightness category is defined as highest third of possible pixel intensity values.



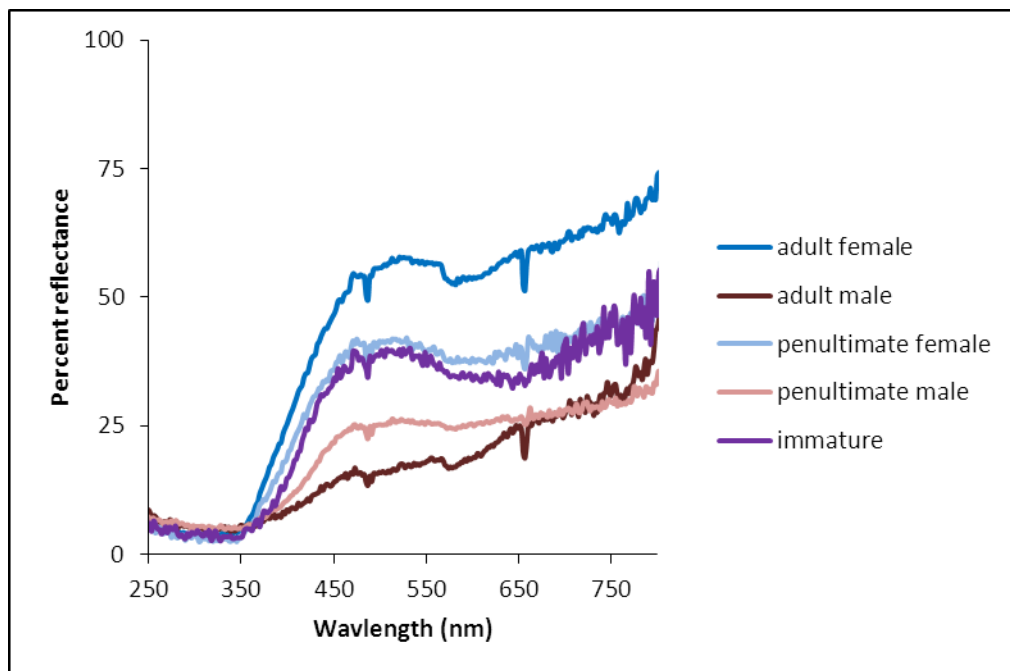
**Figure 1.16** Percent area of given body parts (in *M. vatia*) in brightest category at 375 nm without blocking filter  
Brightness category defined as highest third of possible pixel intensity values



**Figure 1.17** Percent area of given body parts (in *M. vatia*) in brightest category at 365 nm without blocking filter. Brightness category is defined as highest third of possible pixel intensity values.



**Figure 1.18.** Percent area of given body parts (in *M. vatia*) in brightest category at 400 nm without blocking filter. Brightness category is defined as highest third of possible pixel intensity values.



**Figure 1.19.** Reflectance measurements from *M. vatia* at various life stages. Multiple individuals for each sex and life stage were averaged together to produce these readings.

## CHAPTER 2: Effects of Freezing on Fluorescence

### INTRODUCTION

Some spiders exhibit externally-expressed fluorescence. Although this fluorescence intensity can in some cases be dramatic, it can also vary widely both within (Chapter 1) and between species (Andrews, Reed, and Masta 2007). All spiders possess fluorophores in the hemolymph, however only some spiders can fluoresce in a way which is externally visible.

When evaluating spiders for externally-expressed fluorescence, we used instrumentation to capture photographs of fluorescence emission excited by wavelengths that excite hemolymph fluorophores (Chapter 1 methods). These hemolymph fluorophores are likely the same fluorophores as those which cause externally-visible fluorescence. This is because we have observed visible fluorescence of spiders illuminated by the same wavelengths that excites the fluorophores. (Chapter 1: Figures 1.4, 1.5). Since we know little about the chemistry of these fluorophores, we could not predict whether any part of the storage or photography process could change the fluorescent properties of the specimens. To ensure biologically-relevant results, we did not want to inadvertently alter the spectral properties of the fluorophores or the tissues containing them. We thus designed an imaging protocol to replicate what the spiders would experience in nature as closely as possible.

However, we were not able to measure fluorescence in living spiders. This was not possible for several reasons. First, the fluorescence imaging of a spider takes

several hours. During this time, the spider needs to remain motionless and in a precise orientation to ensure that all spiders are measured comparably. This requires that we use pins to hold the spiders in a standardized position. No living spider will remain motionless, even while pinned, for the time necessary to capture all the images. Second, it was not possible to image spiders solely during periods when they were available in the wild. Therefore, it was necessary to collect spiders or rear them in captivity, and preserve them for later analysis. Finally, specimens had been collected over the course of several years and needed to be stored until the current imaging system was available for use.

Freezing at  $-80^{\circ}\text{C}$  was determined to be the best option for preservation, but it was never conclusively shown that freezing did not affect fluorescence. We therefore needed to perform a series of experiments to specifically determine whether fluorescence measurements taken from previously-frozen spiders were consistent with measurements taken from living spiders. To address this issue, we analyzed images of spiders that were anesthetized with carbon dioxide and then re-measured the same spider after freezing.

There was the additional concern that carbon dioxide anesthesia may itself have an effect on fluorescence. Spiders have an open circulatory system (Foelix 2010), so gasses introduced via the respiratory system are dissolved in the hemolymph and bathe the spiders' tissues more or less directly (Foelix 2010). If this carbon dioxide were to directly or indirectly affect the fluorophores, changes in fluorescence could be seen. To

ensure that this was not the case, some spiders were imaged after immobilization without anesthesia.

Finally, we needed to determine whether changes in fluorescence occurred at the moment of thawing or while the specimen is frozen. If changes in fluorescence occurred at the moment of thawing, an increased time spent frozen would not increase the change in fluorescence, and freezing would then be a good way to preserve specimens indefinitely. If, however, the change in fluorescence occurred over the entire time a specimen was frozen, a longer time frozen would correspond to an increased change in fluorescence. To test this, we performed an analysis of the *M. vatia* data from Chapter 1 to determine if the length of time a spider was frozen has any bearing on the brightness of its fluorescence.

## MATERIALS AND METHODS

### **Specimens Used**

Freezing trials were performed on two different groups of spiders. The first group consisted of what we termed the “white” thomisids, because members of these genera are generally white in color. They also possess variable color-changing abilities and a unique suite of pigments (Insausti and Casas 2008; Insausti and Casas 2009). White thomisids are also among the most brightly fluorescent of the spiders previously studied and their fluorescence emanates entirely from the cuticle rather than setae (Andrews, Reed, and Masta 2007). For the white thomisids, we used *Misumena vatia*

specimens and specimens from the *Mecaphesa* genus. The *Mecaphesa* specimens were penultimate female individuals, so it was not possible to identify them to species. However, their overall appearance and fluorescence was consistent with adult female *Mecaphesa* specimens.

The second group consisted of a more taxonomically diverse assemblage, with *Araneus diadematus*, *Phidippus audax* (a salticid with only fluorescent setae), and *Bassaniana utahensis* (a dark species of thomisid). These spiders all exhibit less fluorescence than the white thomisids. They also all possess fluorescent setae, with or without fluorescent cuticle patches.

### **Carbon Dioxide Anesthesia**

The first set of experiments involved comparing fluorescence intensity measurements in spiders before (under anesthesia) and after freezing.

A total of 13 individuals were used for this experiment: 3 adult female *Misumena vatia*, 3 penultimate female *Mecaphesa* sp., 4 adult female *Araneus diadematus*, 1 penultimate female *A. diadematus*, 1 adult male *A. diadematus*, and 1 adult female *Phidippus audax*. Due to the time of year that we conducted this study (late fall) it was difficult to find live individuals of the Thomisidae family, hence the low sample sizes (and use of penultimate, rather than adult, *Mecaphesa* specimens). We maintained all specimens alive in the lab as described in Chapter 1 methods until the day of the trial.

For the first part of the freezing study, we anesthetized each spider using carbon dioxide obtained from the sublimation of dry ice. We used carbon dioxide for anesthesia



because even though it is not a very potent anesthetic in spiders, solvent-based anesthesia (ether, ethanol) (Cooper 2011) had the potential to leach fluorophores from the spiders.

After the spider was anesthetized (generally within 20-30 minutes), we performed spectrometer measurements and fluorescence photography. Because the spiders tended to recover from carbon dioxide anesthesia very quickly, even after extended periods of exposure to carbon dioxide, we could only image specimens at 340 nm (117 second exposure) and 365 nm (38 second exposure). These exposures were chosen because they were the two wavelengths closest to the peak excitation of the spider fluorophores (330 nm).

Images were also only taken with the 450 nm blocking filter engaged. Using the blocking filter only allowed visualization of the brightness of emission spectra above 450 nm, and therefore had the potential to make comparisons with the other fluorescence studies more difficult. However, if the images were extremely bright (as the white thomisids tended to be), we needed to ensure that the images would not be washed out, possibly obscuring subtle differences between the images. Reducing the light arriving at the camera with the blocking filter helped accomplish this.

After the first set of measurements, we euthanized each spider by freezing at -80 °C. The same imaging procedures were then repeated after the spider had been frozen for at least a day, and then allowed to thaw.

Image analysis was conducted only on the dorsal aspect of the abdomens as described in Chapter 1 methods.

### **Immobilization**

To ensure that the use of carbon dioxide anesthesia did not cause a change in fluorescence, we performed another freezing comparison study using immobilization instead of carbon dioxide anesthesia. We used 2 adult female *Mecaphesa sp.* for this study. First, each spider was cooled in a refrigerator at 4 °C for 10 minutes. We then placed the spider dorsal-side up onto a piece of black electrical tape that had been affixed to a surface with the sticky side facing upward. After the entire spider was carefully adhered to the tape, pictures were taken as described for the carbon dioxide anesthesia measurements. After imaging, each spider was frozen at -80 °C and maintained there for several days before re-imaging.

### **Freezing Duration Study**

To determine whether longer freezing times caused a greater change in fluorescence, we also performed a simple linear regression of the fluorescence brightness versus the duration of freezing at -80 C. We performed this regression on a pooled data set for all *M. vatia* from the fluorescence study for which freezing duration was known (n = 30).

## RESULTS

### Carbon Dioxide Anesthesia

In dark thomisids, there were only small changes in fluorescence intensity between when a spider was alive and anesthetized, versus after it had been frozen at -80° C. Some *A. diadematus* specimens decreased in brightness after freezing, but the fluorescence brightness of *P. audax* and *B. utahensis* specimens did not change substantially (Figures 2.1, 2.2, 2.3). In a representative dark-pigmented spider, reflectance readings were similar between anesthetized and frozen individuals (Figure 2.4).

In contrast, freezing caused an increase in fluorescence brightness in all white thomisids at 340nm and 365 nm (Figures 2.1, 2.5, 2.6). Spectrometer measurements also revealed increased reflectance between 350 and 450 nm, after freezing a representative white thomisid (Figure 2.7). We also assayed if the length of time that a spider was frozen affected its subsequent fluorescence brightness. We found that the brightness of *M. vatia* specimens did not show an increased change with increased time spent frozen (Figure 2.8).

In three *M. vatia* specimens that were analyzed and two *Mecaphesa*, the relative fluorescence intensity was greater at 365 nm excitation than at 340 nm excitation (Figure 2.5). This differs from the pattern observed when the individuals were alive, but anesthetized, in which the fluorescence emissions were similar at the two different excitation wavelengths.

### **Immobilization**

The fluorescence intensity of the *Mecaphesa* specimens was relatively lower when they were immobilized compared to after they had been frozen (Figure 2.9). Both sets of measurements are comparable to specimens which were treated with carbon dioxide anesthesia.

### **Freezing Duration**

*M. vatia* specimens that had never been anesthetized (from the Chapter 1 study) did not display brighter fluorescence at 365 nm, as was seen after freezing the specimen (Figure 2.10).

### **DISCUSSION**

The purpose of this study was to determine whether preserving spiders by freezing at -80 °C changes their external fluorescence intensity compared to when they were alive. We found that freezing affects fluorescence brightness in some, but not all spiders. Freezing had negligible affect on the fluorescence intensity of the orb-weaving spider *Araneus diadematus*, the jumping spider *Phidippus audax*, or the crab spider *Bassaniana utahensis* as compared to when these spiders were alive. In dark thomisids, small changes are seen in fluorescence intensity (Figures 2.1, 2.2, 2.3). Similar small, but negligible, differences are seen in reflectance measurements (Figure 2.4). It is possible that subtle differences in the imaging protocol could be the cause. This makes sense, because fluorophores are probably contained within non-living materials (cuticle and setae) in the darker-pigmented spiders. These materials are not likely to be affected by freezing.

In the white thomisids, however, fluorescence intensity increased dramatically after freezing (Figure 2.1, 2.5, 2.6). In some *M. vatia* specimens, the brightness of fluorescence almost doubles after freezing, while in other *M. vatia* individuals there is closer to a 30% increase in brightness (Figure 2.5). These same patterns hold true for *Mecaphesa*. In reflectance measurements of a representative individual, an increase in post-freezing reflectance localized near the fluorescence emission can be seen (Figure 2.7).

In white thomisids, fluorophores are not contained within setae. Rather, they are found either within the cuticle itself or directly beneath it with a transparent cuticle allowing fluorescence to be visible. If fluorophores are found in underlying living tissues, these tissues could be more greatly affected by freezing than the non-living tissues containing fluorophores in dark-pigmented spiders. We discuss below several possibilities of how living tissue containing fluorophores in the white thomisids could cause the change in fluorescence intensity after freezing.

#### **Tissue damage due to Freezing**

Living tissues are sensitive to freezing, as cells can be easily ruptured by ice crystals. If cells containing fluorophores in white thomisids were ruptured by freezing, the cells could release additional fluorophores into the surrounding hemolymph. If the hemolymph were then exposed to excitation wavelengths via a transparent cuticle, increased fluorescence intensity could be seen.

Trauma to the tissue due to freezing may also release additional chemicals (such as reactive oxygen species) that alter the chemical environment, perhaps via pH changes. These changes in the chemical environment might then influence the fluorophores' fluorescent signature.

Additionally, if freezing was causing damage to the tissue containing fluorophores, we would expect that the change occurs at the moment of freezing, rather than over time. Solid crystals of frozen liquid should not change over time, assuming they are maintained at the same temperature.

The data seem to support the tissue damage hypothesis. White thomisids (with fluorophores contained in living tissues) had a greater change in fluorescence than darker-pigmented spiders. Also, the length of time spent frozen had no bearing on the fluorescence intensity of a spider (Figure 2.8).

### **Changes in Chemistry due to Carbon Dioxide Anesthesia**

Another possible cause of the change in post-freezing fluorescence intensity could be due to the use of carbon dioxide anesthesia. The spiders in the freezing study, especially the white thomisids, had to be exposed to carbon dioxide for extended periods. Often we had to keep them in the anesthesia apparatus for two or three periods of 30 minutes each, with them recovering from anesthesia after 5 minutes in fresh air between treatments. These spiders were quite resistant to carbon dioxide compared to other organisms. The recommended carbon dioxide exposure time is only several minutes for most terrestrial arthropods (Cooper 2011).

An excess of carbon dioxide in the tissues of a spider could potentially affect either the fluorophores or the metabolic processes producing them, via acidification. If following anesthesia there had been enough time to purge the spider of carbon dioxide prior to freezing, these effects could be reversed, leading to higher post-freezing levels of fluorescence intensity.

Many fluorophores can be affected by a change in pH. The effects can be wide-ranging, with some fluorophores increasing emission intensity at low pH, while others decrease intensity. Some fluorophores even shift their excitation or emission peaks (Sameiro and Goncalves 2009).

If lowered pH (or increased carbon dioxide in general) led to increased fluorescence, we would expect that when spiders were merely immobilized, there would be no difference in fluorescence between pre and post-freezing measurements. This seems to be the case, as fluorescence in immobilized spiders change to a similar degree as those anesthetized with carbon dioxide (Figures 2.5, 2.9). Thus, we can conclude that carbon dioxide is an unlikely causal agent for the increase in fluorescence intensity after freezing.

#### **Relative Changes Between 340 nm and 365 nm Exposures**

Fluorophores extracted from the hemolymph of *M. vatia* have a peak excitation at 330 nm. Therefore we expect that fluorescence intensity at an excitation of 365nm would be lower than at an excitation wavelength of 340 nm. However, we see that after freezing, *M. vatia* specimens have brighter fluorescence intensity at 365 nm (Figure 2.5).

This is more difficult to explain than the increased overall fluorescence after freezing. However, there are several possibilities for why this change might occur.

One reason for the change in relative brightness could be an altered chemical milieu surrounding the fluorophores, caused by a change in pH or a rupturing of cells following freezing. This might change the peak excitation of these fluorophores from 330 nm to one closer to 365 nm.

Another possible reason for this difference could be technical in nature. Perhaps there are additional fluorophores that were not isolated by the fluorophore extractions from the hemolymph. Another fluorophore may be present in *M. vatia* and other thomisids that has an excitation peak closer to 365 nm. It is possible that this other fluorophore is more resistant to photochemical bleaching than the fluorophore with peak excitation at 340 nm, and hence display relatively brighter fluorescence when excited with 365 nm wavelengths.

However, there are several reasons why this hypothesis does not seem likely. First, in prior attempts at fluorophore extraction from spiders, the entire spider abdomen was ground up and extracted. While the majority of the extract would constitute the hemolymph, if other fluorophores were present in the cuticle or setae, they should have also been extracted. Second, we have found that these fluorophores exhibit a fair amount of stability against degradation, and retain their spectral properties after storage for multiple days.



It is also possible that a fluorophore is present in spiders that cannot be extracted by the solvent (ethanol) that we used for fluorophore extraction. However, when developing protocols for fluorophore extraction multiple different solvents were tried, and ethanol proved to be the best solvent for extraction of fluorophores from spiders.

Finally, the change in relative fluorescence intensity could simply be an anomaly due to inconsistent use of imaging equipment. This seems most likely, as the sample size was small ( $n = 3$ ), and this pattern is not seen in *M. vatia* specimens that were frozen but not previously anesthetized (Figures 1.6, 1.9, 2.10).

#### **Implications for Fluorescence Study**

Freezing has an effect on external fluorescence in some spiders. Although this effect is small in darker-pigmented spiders, fluorescence in white thomisids tends to increase a substantial amount after freezing. Certainly, additional freezing experiments need to be undertaken to better understand the effects of freezing on fluorescence, especially in light colored thomisids. In the meantime, the unpredictable nature of fluorescence changes due to freezing could cause complications in comparing fluorescence intensity among light colored thomisid individuals, such as *M. vatia* and other spiders that are darker in color.

If the effects of freezing were truly as unpredictable as the freezing study suggests across all sexes and life stages, there would be considerable variance and possibly no way to distinguish males from females based on fluorescence. However, in

every comparison of male and female *M. vatia*, the females were dramatically brighter (Chapter 1 Figures). It thus seems most likely that freezing affects fluorescence in males and females in a similar way. A larger sample size, and inclusion of spiders of different sexes and life stages, would help to determine the variance among fluorescence intensity measurements of when an individual is alive versus after it has been frozen.

### **Conclusions**

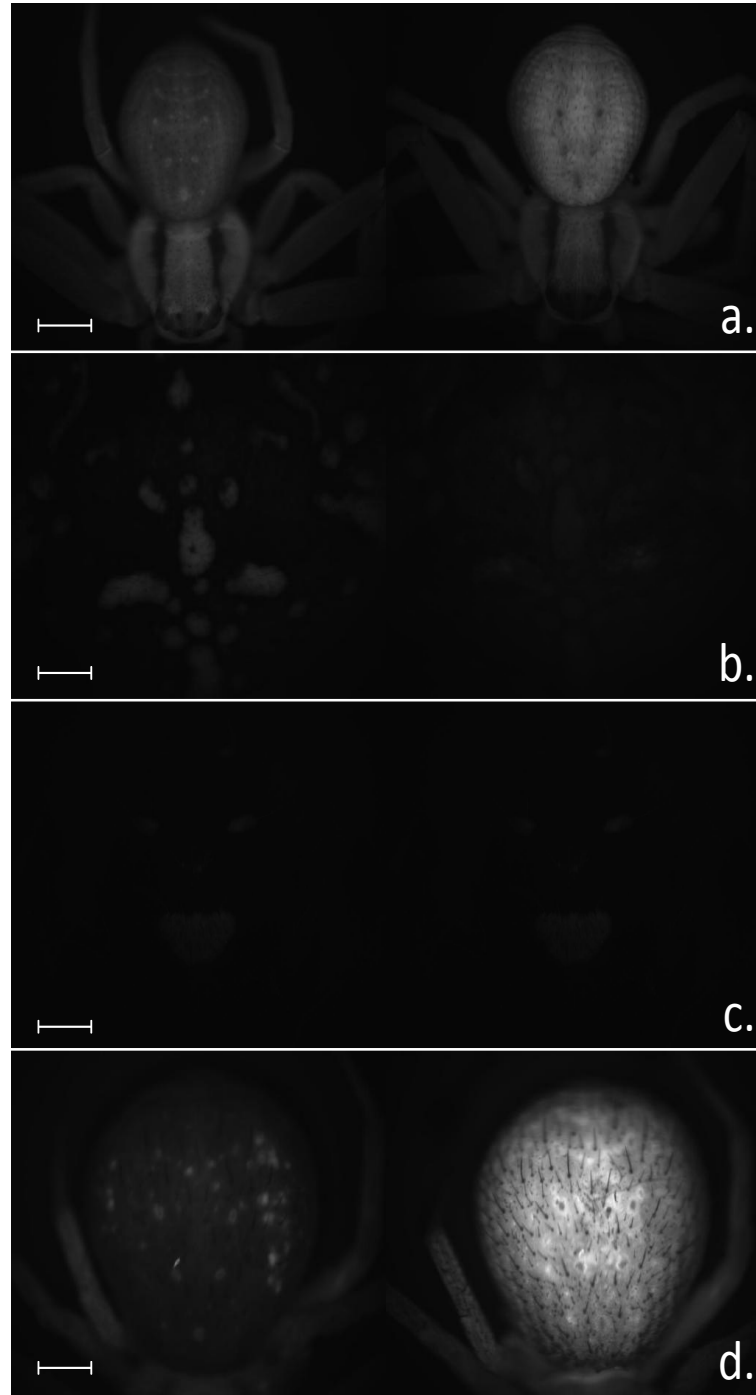
Freezing affects external fluorescence in a variable manner across spiders. In darker-pigmented spiders, there are in general negligible changes between anesthetized and frozen spiders. However, in white thomisids, there are substantial increases in fluorescence intensity. The differences in fluorescence between these two groups may be related to white thomisids possessing fluorophores in living tissue, whereas darker-pigmented spiders contain their externally-visible fluorophores in non-living cuticle and setae.

White thomisids thus probably change in their fluorescence intensity because of damage done to the fluorophore-containing tissue during freezing. This change seems to occur at the moment of freezing, as fluorescence intensity does not change further after increased time spent frozen.

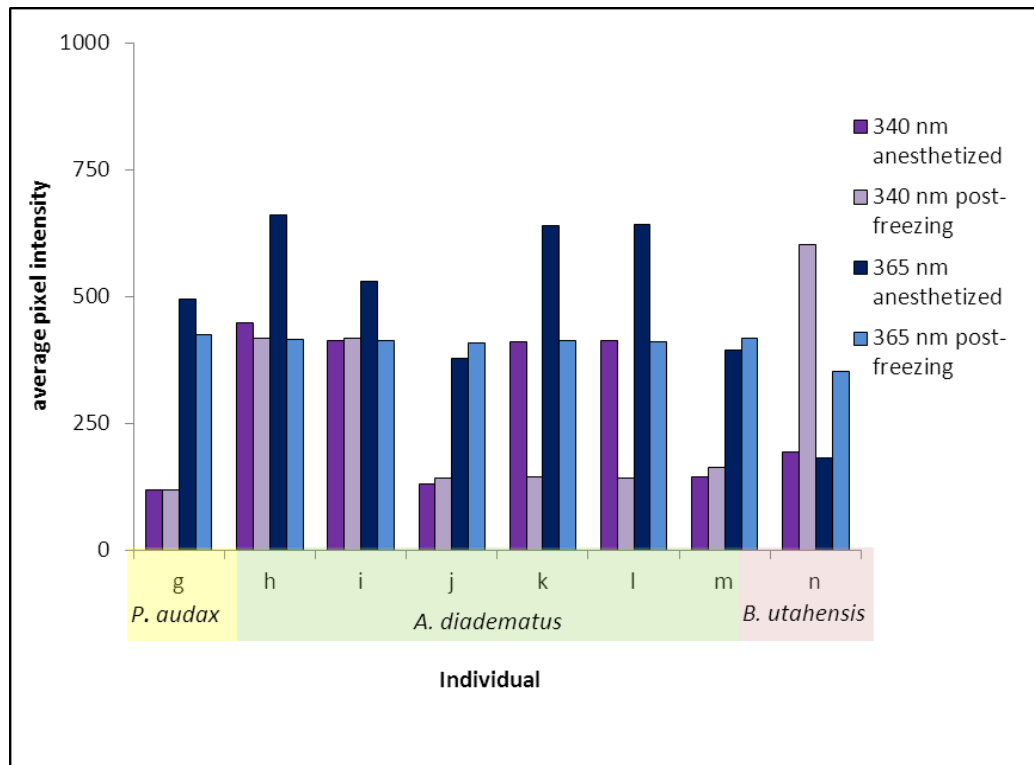
There is a trend for fluorophores in post-freezing white thomisids to have longer excitation wavelengths than anesthetized specimens. However, it is likely that this is an anomaly, as the same pattern is not seen in other spiders measured after freezing.

Finally, wider-ranging freezing studies need to be undertaken to assess the extent to which fluorescence intensity changes after freezing across sexes, life stages, and species. This will help inform future fluorescence studies seeking to understand fluorescence in spiders.

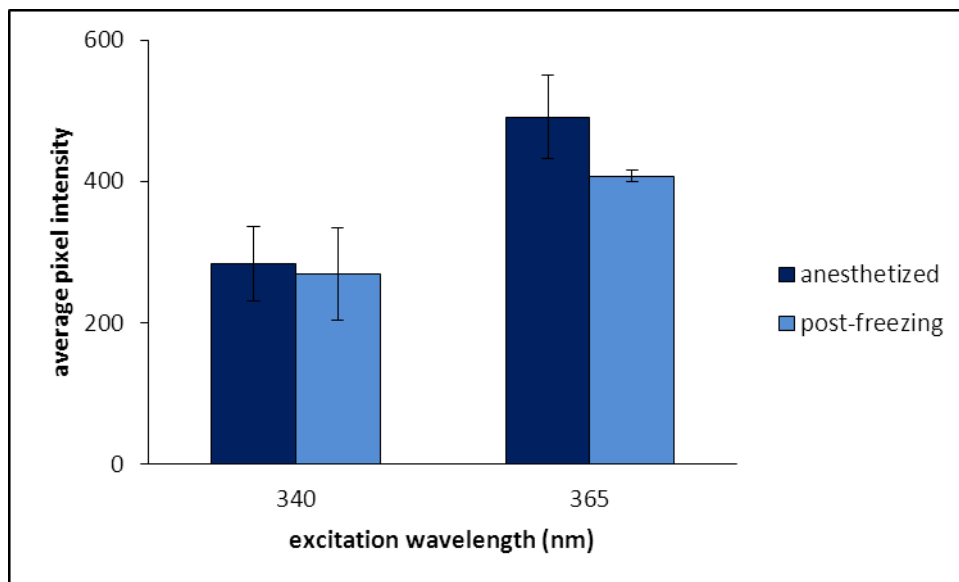
## FIGURES



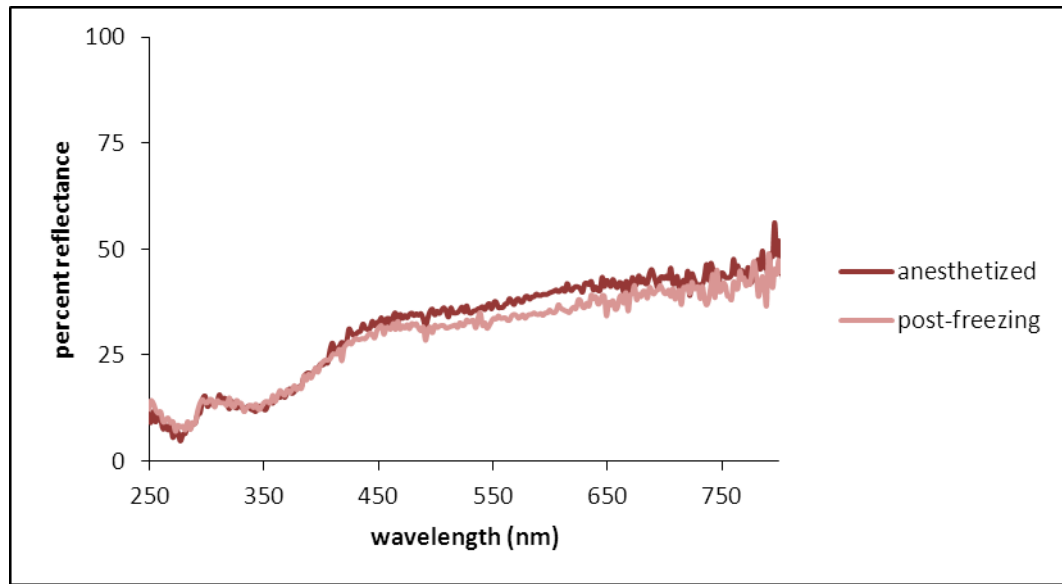
**Figure 2.1.** Photographs taken at 340 nm without blocking filter before freezing (left), and the same individual photographed after freezing (right). Individuals are (a) *M. vatia*, (b) *A. diadematus*, and (c) *P. audax*, and (d) *Mecaphesa* sp. All individuals were anesthetized for the left panel except for (d), which was immobilized. Bar = 1mm.



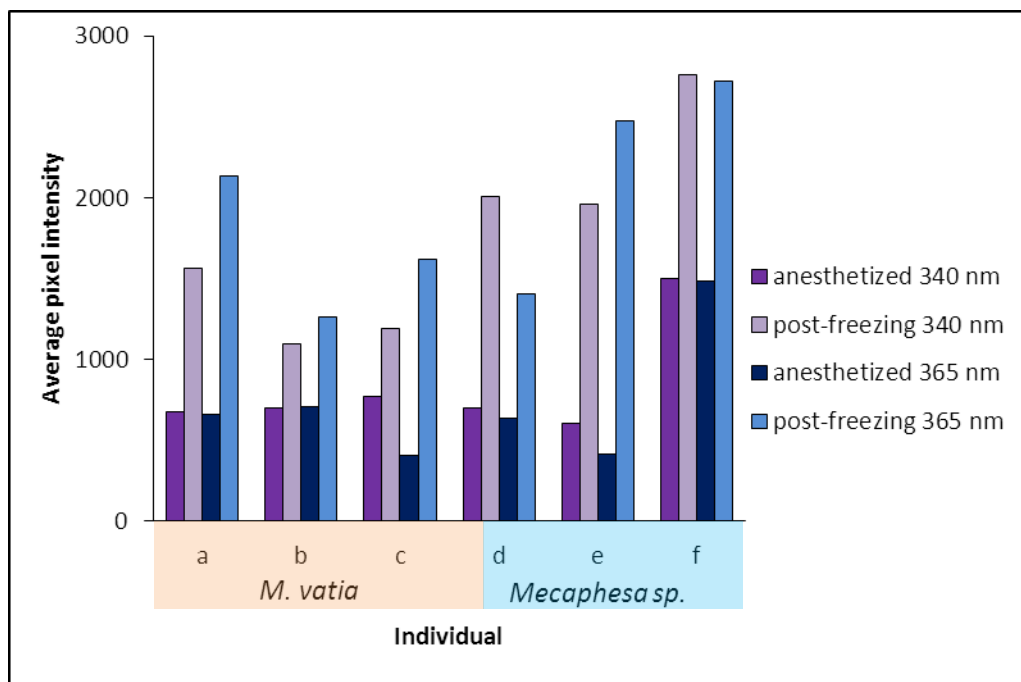
**Figure 2.2** Average pixel intensity for abdomens of 1 *P. audax* specimen, 6 *Araneus diadematus* and 1 *Bassaniiana utahensis* specimen before (anesthetized) and after freezing. Individuals were given the identifying letters g-n. Images were taken at 340 nm and 365 nm with the blocking filter.



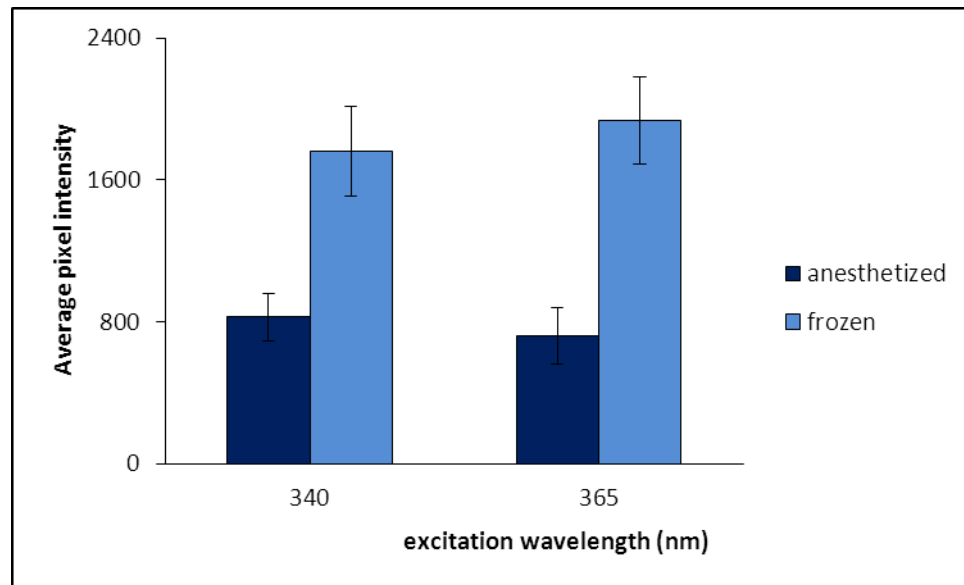
**Figure 2.3.** Average pixel intensity for all dark-pigmented spiders (n = 8) at 340 nm and 365 nm excitation before and after freezing. The blocking filter was used.



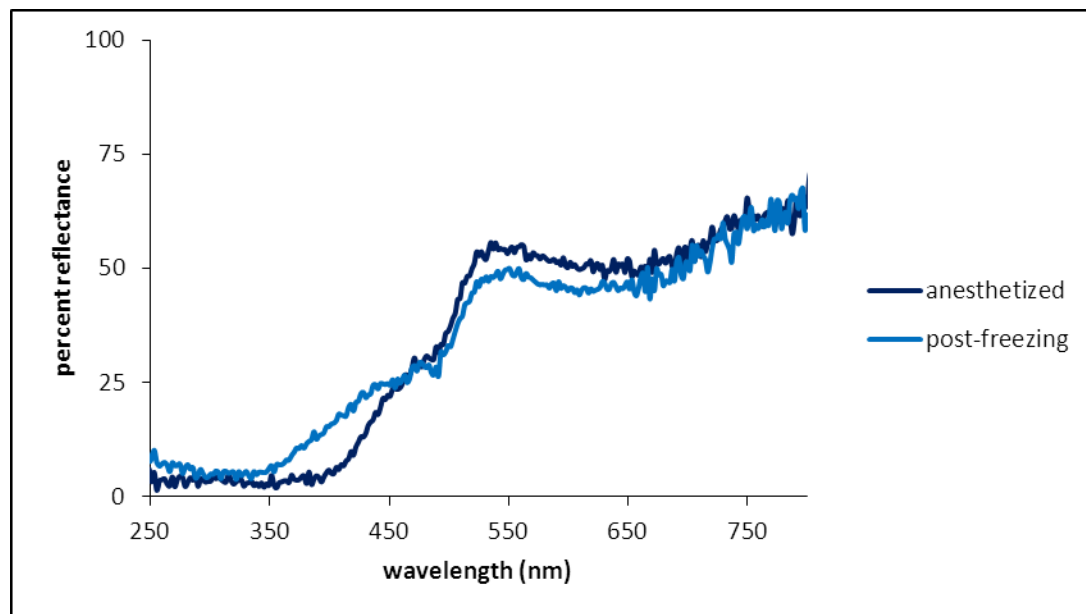
**Figure 2.4.** Reflectance measurements taken from adult female *P. audax* under anesthesia and after freezing



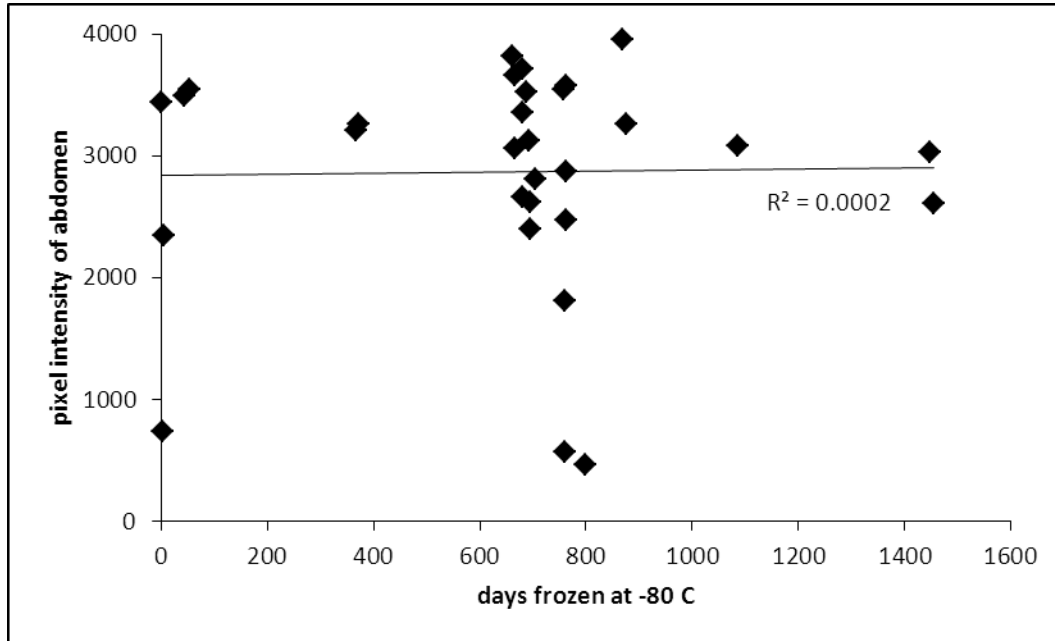
**Figure 2.5.** Average fluorescence intensity for abdomens of 3 *Misumena vatia* specimens and 3 *Mecaphesa sp.* specimens before (anesthetized) and after freezing. Individuals are given the identifying letters a-f. Images were taken at 340 nm and 365 nm with the blocking filter.



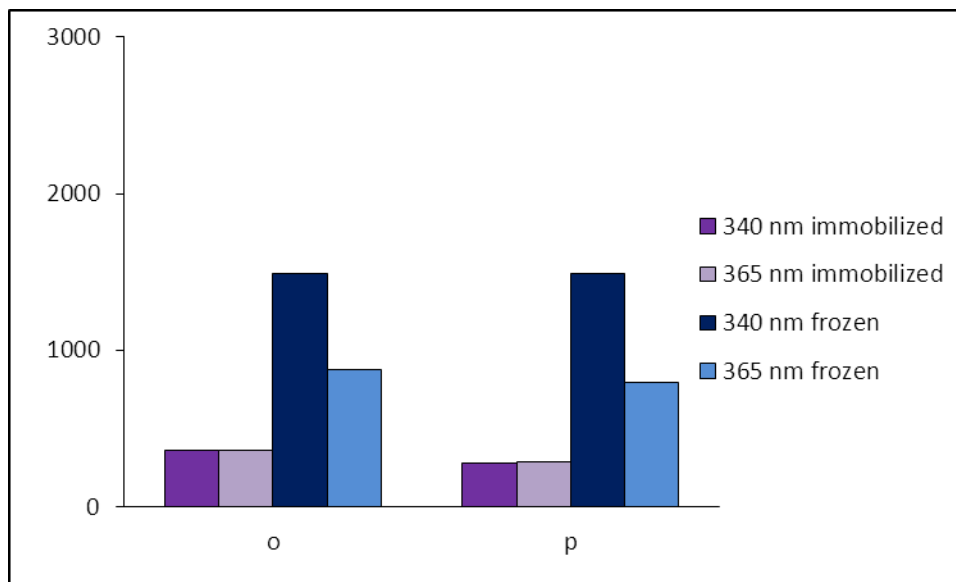
**Figure 2.6.** Average pixel intensity for all white thomisids ( $n = 6$ ) at 340 nm and 365 nm excitation before and after freezing. Blocking filter was used.



**Figure 2.7.** Reflectance measurements taken from adult female *M. vatia* abdomen during anesthesia and after freezing.

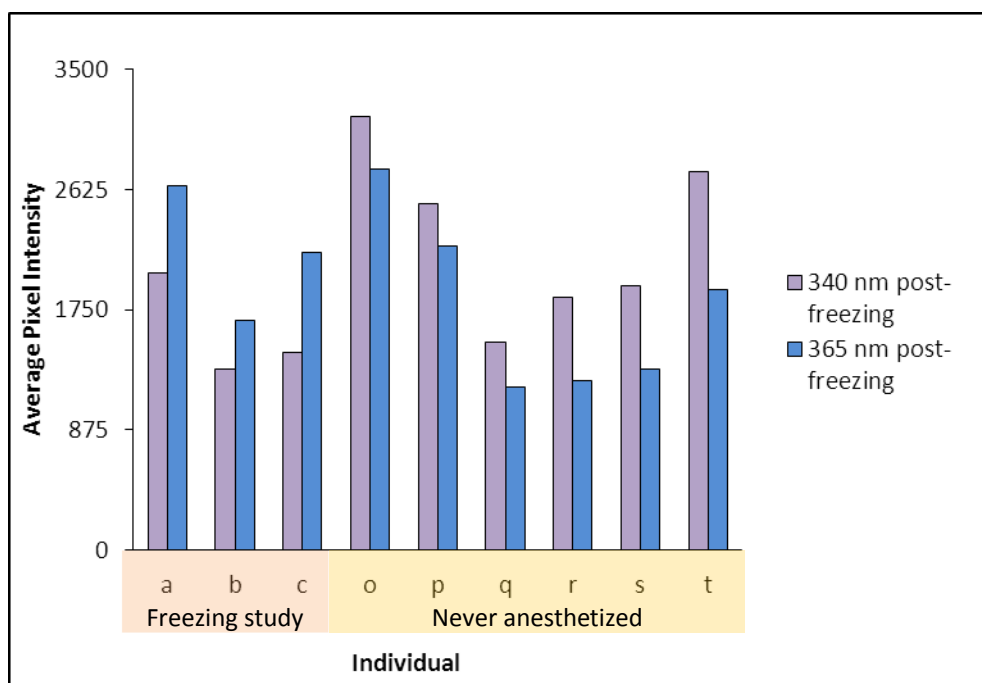


**Figure 2.8.** Graph depicting a linear regression of average abdomen brightness versus the number of days frozen for all *M. vatia* specimens used in fluorescence study. All measurements were taken with 340 nm excitation light without the blocking filter.



**Figure 2.9.** Average pixel intensity for abdomens of 2 *Mecaphesa* specimens with readings taken while immobilized and after freezing. All images were taken with the blocking filter.





**Figure 2.10.** Comparison of average fluorescence brightness in adult female *M. vatia* abdomens after freezing. Individuals a-c were used in the freezing study and anesthetized and photographed prior to freezing. Individuals o-t were frozen but not previously anesthetized. Photographs were taken at 340 nm and 365 nm with blocking filter.

## CHAPTER 3: Differences in Fluorescence across Sexes and Life Stages in *Araneus diadematus*

### INTRODUCTION

Although all spiders possess fluorophores in their hemolymph, the amount of external fluorescence exhibited, and how it is expressed, varies between species (Andrews, Reed, and Masta 2007). Externally visible fluorescence can be due to fluorophores either within or directly beneath the outer cuticle. Fluorophores may also be sequestered within the setae of the animal. It is possible that the presence or absence of fluorescent cuticle and setae could vary within a species, but this has not previously been determined.

Fluorescence expression varies in different sexes and life stages in *Misumena vatia*, a flower-dwelling ambush predator (see Chapter 1). This chapter will explore how externally expressed fluorescence varies between sexes and life stages in a web-based predator, *Araneus diadematus*. This orb-weaving spider possesses ecological characteristics very different from those of *M. vatia*, although both possess externally-expressed fluorescence.

*A. diadematus* is a common species often associated with human habitation. It has a Holarctic distribution and builds large (1 m in diameter), conspicuous orb webs. These webs have made *A. diadematus* one of the most readily-recognized garden spiders. Adults possess a characteristic cross pattern on the dorsal aspect of the abdomen which has given them the common name of “garden cross spider”.

*A. diadematus* are strictly web-based predators. They sit in the hub of their large webs and wait for prey (usually flying insects) to become ensnared in the sticky threads of the web. Vibrations from the struggling prey are transmitted through the silk threads of the web to the hub. The spider intercepts the vibrational signals and then moves to capture and consume the prey (Zschokke 2002; Foelix 2010). Although there is a highly developed vibrational sense, vision in *A. diadematus* is poor (Foelix 2010).

Feeding and behavior vary across sexes and life stages in *A. diadematus*. Adult females and penultimate individuals use the aforementioned large webs for food capture. However, earlier instars weave smaller webs closer to the ground. They also capture much smaller food items, such as small flies and gnats. In second instars (the first molt after leaving the egg sac), spiderlings do not eat or spin webs, but rather stay in groups of their littermates, waiting for wind on which to disperse (Burch 1979). Adult males do not spin webs or consume prey.

Because the ecology of these spiders changes during the course of their lifetime, it is possible that expression of fluorescence also changes during their lifetime, making *A. diadematus* a good candidate for the examination of how fluorescence expression may be influenced by ecology. Thus, one goal of this project was to examine fluorescence differences between the sexes in light of these ecological differences.

The coloration of *A. diadematus* may also vary across life stages, although this has not previously been systematically examined. It is known that the earliest instar

spiderlings possess yellow and black coloration, which changes to a mottled brown pattern with subtle yellow and white patches later instars (Cushing and Ubick 2009). Therefore, we sought to determine if the changes in coloration are concordant with changes in fluorescence during the different instars, including potential variation between the sexes at sexual maturity.

To address external fluorescence changes over the lifetime of *A. diadematus*, we reared spiders to each instar and captured fluorescence photographs. We then analyzed the photographs to determine average fluorescence intensity and compared each sex and instar.

Additionally, we found that in *A. diadematus* there is a great deal of variance in the presence of fluorescent cuticle and setae among the different life stages. Thus, an additional purpose of this study was to examine the relative intensity of these two fluorescent materials and how they might impact the overall fluorescent signal of an individual. We examined these differences by comparing the fluorescence intensity of these two materials.

## MATERIALS AND METHODS

To assess fluorescence across all sexes and life stages in *A. diadematus*, we needed to first obtain individuals of both sexes and at all life stages. However, it is not possible to identify the developmental stage of an *A. diadematus* individual by simply examining the spider. Therefore, to be certain of the life stage of each specimen, it is

necessary to rear the spiders in captivity, and carefully record each molt stage as individuals pass through them.

Spiders develop via discrete stages, each ending with the molting of the exoskeleton which transitions the spider to the next developmental stage. If spiders are reared in the lab and molting is observed and recorded, determining the exact developmental stage of spiders is relatively straightforward. However, rearing orb weaving spiders in a laboratory is fraught with difficulties, and very few spiders typically survive until their final adult stage. We were able to raise *A. diadematus* through all early developmental stages and freeze them for later fluorescence assessment.

In the winter and early spring of 2010, we collected a total of 11 *A. diadematus* egg sacs from the Portland, OR. Metro Area. Each egg sac was kept in a 40-dram vial, and maintained at room temperature on a 12:12 hour light cycle. There is some evidence that orb weaver spiderlings have a higher rate of survival when they are allowed to cohabitate with and consume conspecifics during the time prior to when dispersal would naturally occur (Burch 1979). Therefore, about a week after each egg sac hatched, we divided the spiderlings up into vials containing ten siblings. A week or two later (often after most of the spiderlings were consumed by littermates), the remaining spiderling(s) were placed individually into 7-dram vials.

We reared the spiderlings to differing stages of maturity, carefully tracking the development of each cohort of spiderlings. Spiders at the following stages of

development were frozen at  $-80^{\circ}\text{C}$  and later assayed for fluorescence intensity: molt 2 (the first molt after emerging from the egg sac), molt 3, molt 4, molt 5, penultimate individuals (both male and female) and adult (male and female). Since few of the lab-reared spiders attained adulthood, we collected supplementary penultimate and adult individuals from the wild. These collections were conducted primarily near the Portland State University campus.

Spider abdomens and cephalothoraxes were imaged following the methods of Chapter 1. The resulting “average pixel intensity” values were used as a proxy for fluorescence brightness. Each individual was also scored for the presence or absence of fluorescent setae and cuticle. Average pixel intensities of setae and cuticle patches were calculated to determine the influence of each of these fluorescent materials on the overall fluorescence brightness. To analyze pixel intensity, we established normality of the data with Shapiro-Wilk tests. Then, we used one-way ANOVAs to evaluate variance between the sexes and molt stages, and post-hoc Tukey’s HSD to establish significant differences between the molt stages.

Two specimens of each life stage were assessed for cuticle and setae brightness (when fluorescent cuticle and/or setae were present at a given life stage). Three cuticle patches about 16 square pixels were used as Areas of Interest (AOIs) and analyzed using the same methods as those used for the entire body. In selecting the regions of cuticle patches to analyze, we attempted to cover the brightness range of fluorescent cuticle

for any given animal, i.e. a “bright” patch, a “medium” patch, and a “dim” patch, chosen in a haphazard manner. Fluorescent setae were chosen and analyzed in a similar manner, except single entire setae were circled and analyzed rather than a patch.

## RESULTS

Emission wavelengths were visible, albeit dimly, in emission wavelength photographs of spiders (Figure 3.1, 3.2). Abdomen and cephalothorax data were normally distributed for the most part. However, for each body part, the data set for one molt stage was not normal: molt 4 for the abdomen (Shapiro-Wilk  $p = 0.006$ ), and penultimate male for the cephalothorax (Shapiro-Wilk  $p = 0.019$ ). Due to the robust nature of ANOVA and Tukey’s HSD, these parametric tests were used despite the slight departures in normality. ANOVA analyses indicated significant differences between molt stages for both abdomen [ $F(7,39) = 14.857$ ,  $p = 2.93 \times 10^{-9}$ ], and cephalothorax [ $F(7,40) = 4.169$ ,  $p = 0.002$ ]. Tukey’s HSD results can be seen on the representative graphs for intensity values.

Most molt stages had similar average levels of fluorescence brightness of their abdomens (Figures 3.1, 3.2, 3.3). However, the abdomens of adult males were significantly brighter than the abdomens of all other molt stages compared in a pairwise fashion ( $p \ll 0.001$  for all). There was a similar trend for fluorescence of the spiders’ cephalothoraxes (Figure 3.4). Adult males possessed significantly brighter fluorescence than all other molt stages except for penultimate females (see Figure 3.4 for  $p$  values).

However, adult males also had high levels of variance in fluorescence in both body parts (figures 3.3, 3.4).

*Araneus diadematus* was found to exhibit fluorescence both from regions of cuticle devoid of setae, and from specific setae. Images captured with 340 nm excitation illustrate this variation in fluorescence expression (figure 3.1, 3.2). However, the presence or absence of fluorescent cuticle and setae varied between and within sexes and life stages. Some developmental stages of *A. diadematus* possessed only fluorescent cuticle. Some individuals only had setae that fluoresced, and others had both fluorescent cuticle and setae. These data is summarized in Figure 3.5. The presence or absence of these two types of fluorescent regions varies even within individuals in a single life stage, but some trends can be seen through development of the spiders.

Fluorescent setae are absent from *A. diadematus* spiderlings upon hatching. Setae begin to develop in molt 3, and by molt 4, most individuals possess both fluorescent cuticle and setae. In molt 5, some specimens have evidently lost their fluorescent cuticle entirely, although during the penultimate stages, it is regained in some individuals. In both penultimate males and females, all three conditions (fluorescent cuticle, setae, or both) can be seen. However, all adult females have both fluorescent cuticle and setae, whereas most males lose their fluorescent setae by maturity.



We also assessed the overall brightness of the abdomen based on whether a given individual possessed fluorescent cuticle, setae or both. This was done to determine whether the manner in which fluorescence is expressed affects the potential visual signal fluorescence can provide. The data were not normally distributed. A Kruskal-Wallis test indicated no significant difference between the overall brightness of the abdomen in individuals that possessed fluorescent setae, cuticle, or both (Figure 3.6). However, there was a trend for individuals with only fluorescent cuticle to be brighter overall than either those with just fluorescent setae or those with both fluorescent cuticle and setae (Figure 3.6).

Individual patches of cuticle and setae were also measured. The data were not normally distributed. Wilcoxon signed rank test indicated no significant differences in brightness in brightness ( $p = 0.280$ ), although the adult male cuticle patches were outliers in this respect (Figure 3.7).

## DISCUSSION

In *A. diadematus*, adult males are brightly fluorescent whereas adult females and all immature spiders are comparatively dimly fluorescent. Although adult males are brighter than all other stages, it is useful to first compare adult males to adult females. These two groups differ substantially in regards to both fluorescence and behavior despite similar developmental stages.

### Differences in Fluorescence Based on Sex

Adult male *A. diadematus* possess much brighter fluorescence than adult females (Figures 3.3, 3.4). Given this difference, we might hypothesize that fluorescence brightness is a trait under selection. Selection can act on a trait in one of several ways. To better understand how this might work, we can think of fluorescence brightness as a trait on a continuum of possible values. First, directional selection can occur if versions of a trait in one area of the continuum are more advantageous than other versions of the trait. If this occurs, we would expect to see decreased variance in that trait in a given population, since only specific versions of the trait allow organisms to remain competitive. We see this in adult female and immature *A. diadematus*. They possess dim external fluorescence with small levels of variance in comparison to the adult males.

On the other hand, diversifying selection can select for multiple versions of a trait, resulting in increased variance in that trait in a population. We might expect this in adult males, if differing levels of fluorescence suit different males based on ecological characteristics. Finally, if a trait is under weak selection or no selection at all, we would also expect to see large amounts of variance in that trait. If a given trait has no bearing on survival or reproduction of an individual, any version of the trait will persist within the population.

Whatever specific effect selection is having on fluorescence brightness in *A. diadematus*, there are clear differences in both brightness and variance of brightness

between the sexes. This suggests differing selective pressures between males and females, and that sexual selection is occurring. Common examples of sexual selection involve sexual signaling. In spiders, males in some species are brightly colored or perform certain displays to attract females (Girard, Kasumovic, and Elias 2011; Wilgers and Hebets 2011). It is unlikely that fluorescence in *A. diadematus* is used in sexual signaling. As previously mentioned, *A. diadematus* have poor vision and use vibrational signals for courting. However, differences in ecology between adult males and females may help explain the differences in fluorescence brightness.

#### **Differences in Fluorescence Based on Web Utilization**

Perhaps the greatest difference between adult males and females in regards to life history is their feeding ecology. Adult females build large orb webs in which to capture prey. Males do not spin webs and do not eat after molting to maturity (Elgar and Nash 1988). This dramatic lifestyle difference could offer insight into why fluorescence differs between the sexes.

There has been a great deal of research pertaining to the interaction between large orb webs, the spiders that weave them, and prey that becomes ensnared. Specifically, it is of interest to know whether visual signals given by webs, web decorations, and the spiders themselves serve as a prey attractant or as camouflage. (See Thery and Casas 2009 for a review). Many araneids have bright coloration and striking patterns of contrast on their abdomens, so many researchers have sought to determine how these specific visual signals affect prey.

Externally- displayed fluorescence has not been evaluated across the Araneidae family. However, fluorescence is not in itself an isolated signal. Both reflectance and fluorescence contribute to a signal that cannot be separated into its component parts by the receiver (Fuchs 2001; Johnsen 2011). Thus, studies examining the overall coloration in araneids are also likely including the part of coloration due to fluorescence, so a brief review of coloration studies is useful in considering possible functions for externally-expressed fluorescence.

It is important to note that araneid coloration studies are usually conducted to determine the function of strongly contrasting patterns on the spider. Measuring contrast in coloration patterns on living organisms is difficult. Spectrometers are useful to determine the spectral characteristics of a given area, but there is a minimum size patch that a spectrometer probe can assess. If patterns consist of many small contrasting patches, it can be very difficult to evaluate the pattern.

New technologies are being developed that can evaluate the reflectance spectrum of every pixel in a photograph, creating a very finely-grained measure of patterning, contrast, and background matching in an organism (Chiao et al. 2011). However, these systems have not yet been used on spiders, as they are expensive and require bright lights and immobility on the part of the specimen. Additionally, although it may be easy to qualitatively describe a pattern, it is more difficult to determine how another organism perceives it.

Researchers take several approaches to evaluate the importance of coloration and contrast in spiders. In some studies, parts of the pattern are masked. The effects of the alteration on other organisms' behavior are then measured. In other studies, patterns are more directly measured and then evaluated based on known characteristics of prey visual systems.

There is some evidence that color contrast in adult female araneids may serve to attract prey. *Argiope bruennichi* possesses bright patches of color on the abdomen. There is evidence that if the contrast in this pattern is reduced, adult females catch fewer prey (Bush, Yu, and Herberstein 2008). A study on *Nephila pilipes* showed that patterns of contrasting colors may even resemble nectar guides when viewed through the eyes of a honeybee (Chiao et al. 2009). Another study in *N. pilipes* combining both spectral analysis and color manipulations showed that patterns of contrasting color in *N. pilipes* specifically exploit low light conditions and the visual systems of moths, as well as diurnal insects (Chuang, Yang, and Tso 2007).

There have been contradictory studies suggesting that the primary function of araneid coloration is to provide camouflage. Hoese et al. (2006) conducted coloration manipulation and presented evidence that coloration in *A. bruennichi* is used for disruptive camouflage. There is also evidence based on artificial web studies that *Gasteracantha cancriformis* has bright coloration that provides camouflage (Vaclav and

Prokop 2006), although another group provided evidence that the coloration may serve as an aposematic signal (Gawryszewski and Motta 2012).

We did not specifically measure contrast while evaluating fluorescence in *A. diadematus*. However, fluorescence does seem to occur in discrete patches on the animal, particularly in the penultimate and adult individuals (figure 3.1). It seems likely that this patterning has some bearing on the ecological function of externally-expressed fluorescence. We will thus focus mainly on differences in fluorescence brightness between sexes and life stages, but it is important to remember that contrast and patterning could be equally or more important than overall fluorescence brightness in these spiders.

If bright fluorescence acted as a prey-attractant in *A. diadematus*, we would expect to see bright fluorescence in individuals which spin large webs. Large webs not only capture more prey but also larger prey, primarily the visually-acute hymenopterans and dipterans (Eberhard 1983). However, adult females which weave large webs do not possess bright external fluorescence. In fact, the dim fluorescence of the females suggests that their coloration may help in camouflage, either to predators, prey, or both. Therefore, it seems unlikely that bright fluorescence is important in prey attraction.

In contrast, adult males possess bright fluorescence, but do not spin webs or consume prey. Therefore, it seems unlikely that male coloration has been selected to

optimize prey capture. Additionally, sexual cannibalism by the female is very common in araneids in general and *A. diadematus* in particular (Elgar 1991; Elgar and Nash 1988). It seems that bright fluorescence may actually be to the detriment of adult males if they are more conspicuous and thus a more obvious meal for the female. However, adult males spend much time searching for webs containing females. Fluorescent patterning may provide a sun-dappled appearance that camouflages adult males as they move through the undergrowth during their searches. A complex signal of contrast and patterning involving both fluorescence and reflectance might play an important ecological role in camouflage for males.

### **Fluorescence through Development**

As in the adult females, all immature *A. diadematus* possess relatively dim fluorescence. Also like adult females, most immature spiders spin webs to capture prey. This suggests that that suppressed fluorescence is advantageous for spiders that spin webs, possibly providing camouflage from prey. However, this hypothesis is problematic. First, not all spiders possess external fluorescence. If it was truly maladaptive for web-weaving *A. diadematus* to fluoresce, it seems more likely that they would completely lack fluorescence. Instead we might hypothesize that some low level of fluorescence is important for web-weaving spiders.

Also, molt 2 individuals do not weave webs or capture prey. At this young stage, spiderlings group together and live off of yolk stores within their bodies until attaining a

larger size. As they inhabit lower, more sun-dappled habitat, we might expect that they possess bright fluorescence like adult males to provide camouflage from predators.

However, molt 2 individuals instead possess dim fluorescence like the large orb-weaving adult females. There is a possibility that dim fluorescence in this case acts to camouflage spiderlings from predators, but in a way different than the adult males. However, this also does not seem likely because molt 2 individuals have very striking yellow and black coloration, and cluster together in large, easily-recognizable groups (Burch 1979). This further calls into question why all molt stages in *A. diadematus* except adult males possess dim, but not absent, external fluorescence.

### **Setae and Cuticular Fluorescence**

Despite substantial variation in the presence or absence of fluorescent cuticle or setae, most life stages possess similar levels of fluorescence (figures 3.1 - 3.4). The exception lies with adult males. Their abdomens fluoresce more brightly on average, and their fluorescent cuticle patches fluoresce more brightly than fluorescent cuticle in other life stages (figures 3.3, 3.7). Many males also lack fluorescent setae.

Many males have eschewed fluorescent setae despite possessing bright overall fluorescence. In fact, individuals with brighter fluorescence in general tend to be those with only fluorescent cuticle (figure 3.6). When cuticle patches and setae are compared for fluorescence brightness, they have the same ability to fluoresce (figure 3.7). Perhaps, then, fluorescent setae are more difficult to produce, leading males to instead focus on producing fluorescent cuticle.



Fluorescent cuticle seems to provide all of the fluorescence brightness that bright adult males require without the addition of fluorescent setae. If a low, but non-zero level of fluorescence brightness is needed for other life stages, we would also expect them to utilize fluorescent cuticle. This is because cuticle can provide the needed level of fluorescence at a low energetic cost. However, we see fluorescent setae present in individuals with low levels of fluorescence.

The question arises of why fluorescent setae are present in *A. diadematus* at all. The answer is not clear. Perhaps fluorophores co-occur in setae with pigments that are important to reflectance, but not fluorescence. It is also possible that the fluorophores result from the catabolism of metabolic components or pigments that are expressed only in some setae. However, we do not know enough about the chemical nature of the fluorophores in setae versus cuticle to draw any definitive conclusions. Contrast and patterning may play a role here, as small patches of fluorescent setae may be a good way to provide precise pinpoints of fluorescence to an overall pattern. Perhaps these subtle patterns consisting of reflectance and dim fluorescence are important to the ecology of non-adult male spiders.

### **External Fluorescence as Photoprotection**

Finally, there is the possibility that externally-expressed fluorescence has photoprotective benefits for those spiders which exhibit it. Spider fluorophores convert potentially-dangerous ultraviolet wavelengths to light in the visual spectrum, so it is possible that they serve such a function. If this were the case, we would expect spiders

regularly exposed to the sun to fluoresce brightly. We would also expect to see fluorophores primarily in the cuticle of such spider. Setae could possibly shade the spider from sun exposure, but it is difficult to imagine a way in which fluorescent setae could have a specific photoprotective effect. Adult males possess bright fluorescence, but are probably not as sun-exposed as females or other life stages. However, low levels of photoprotection may serve to explain why females and immatures have dim fluorescence, rather than no fluorescence at all.

## CONCLUSIONS

*A. diadematus* is a good study system for exploring external fluorescence, as ecology and fluorescence vary with life stage. Adult males possess the brightest externally-expressed fluorescence, while the rest of the life stages and females do not possess bright fluorescence. There is a possibility that fluorescence serves to camouflage adult males as they search for mates, although it is not clear why web-weaving spiders would possess dim, but not absent, levels of external fluorescence. External fluorescence may play a role in patterning and contrast. Dim, fluorescence in web-weaving *A. diadematus* could contribute to an overall pattern providing either a prey-attractive or camouflaging role.

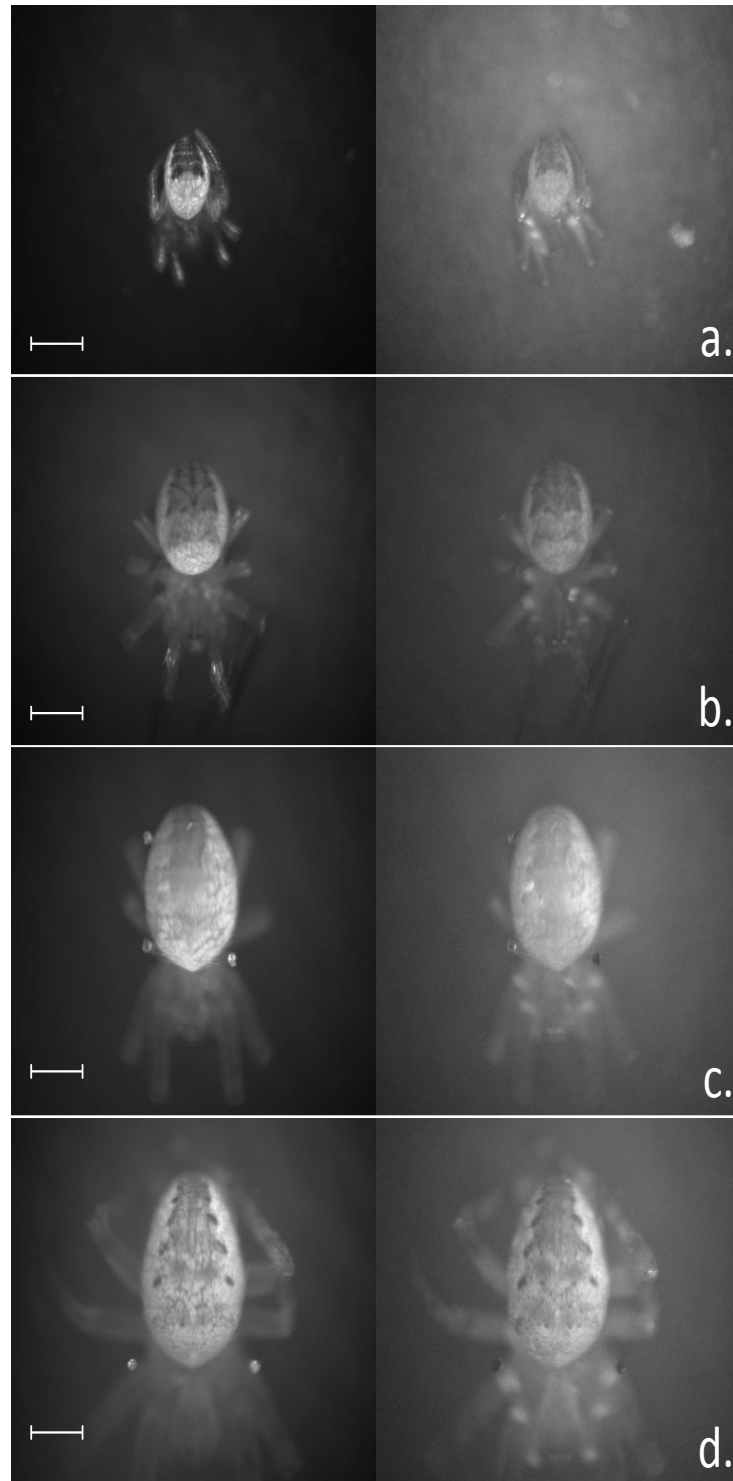
## FUTURE AREAS OF INQUIRY

More detailed work examining coloration across life stages would be useful to help understand the role of fluorescence in the context of overall coloration. Field

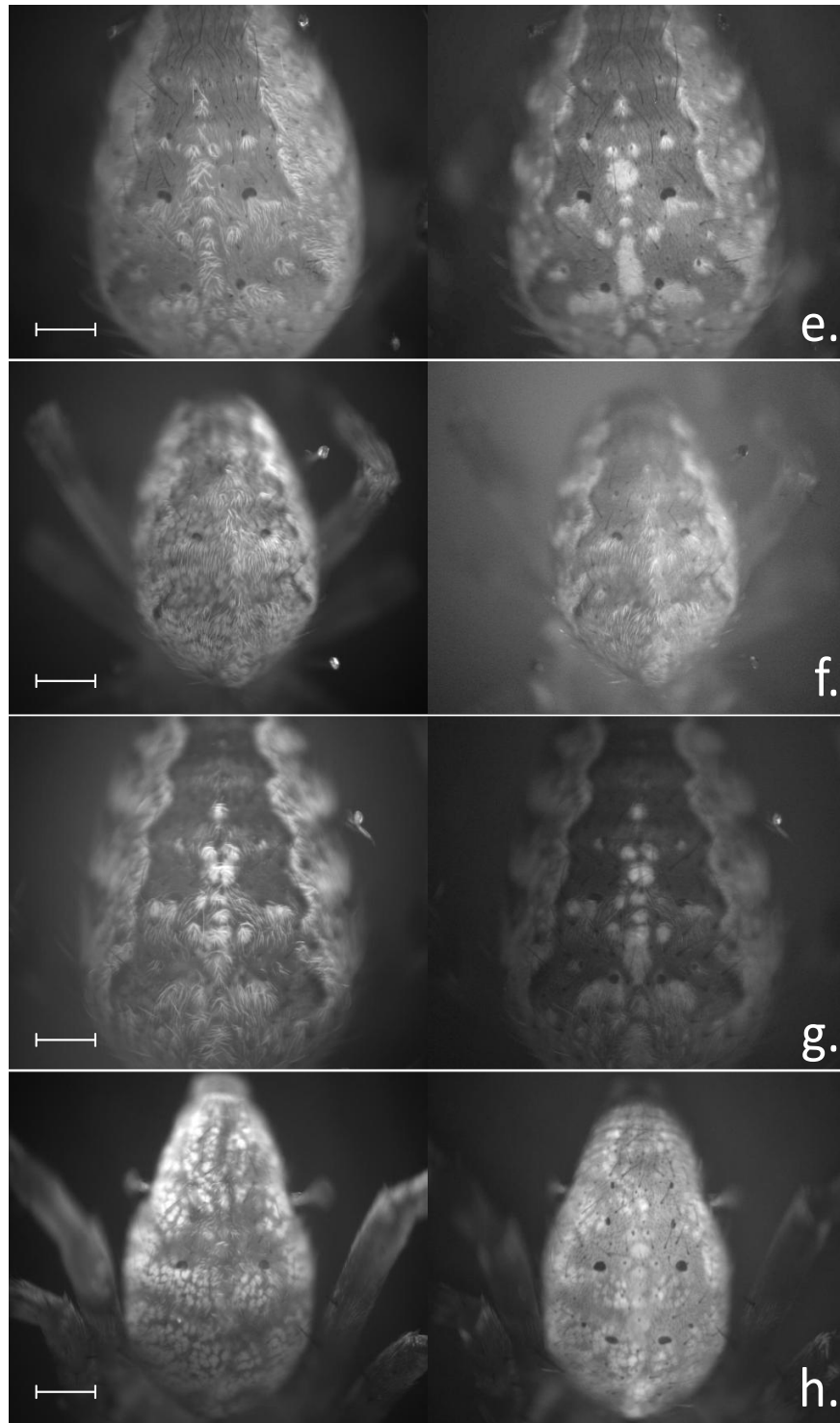
studies could also be valuable in addressing whether experimentally-augmented fluorescence in adult females causes decreased camouflage and prey capture.

Finally, there is the possibility that fluorophores are moved from the hemolymph to setae in an active sequestration process, which could be metabolically expensive. The fact that brightly-fluorescent males increase fluorescence in cuticle rather than setae supports this. However, we do not currently know whether fluorophores found in cuticle are the same as those found in the hemolymph, although they exhibit the same peak excitation wavelengths. Detailed chemical analysis of the fluorophores in the setae would determine this and help elucidate the role of fluorescent setae in this species.

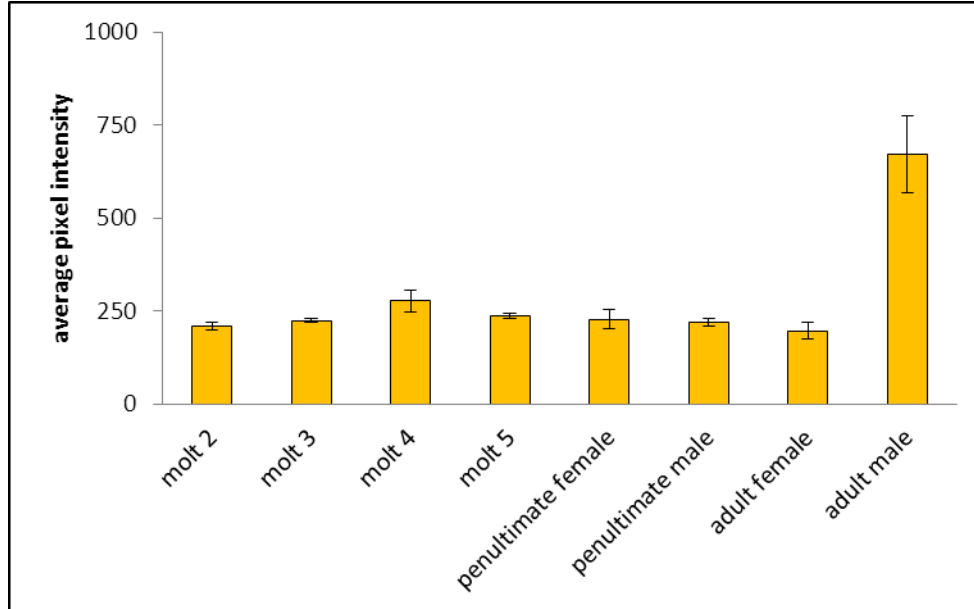
## FIGURES



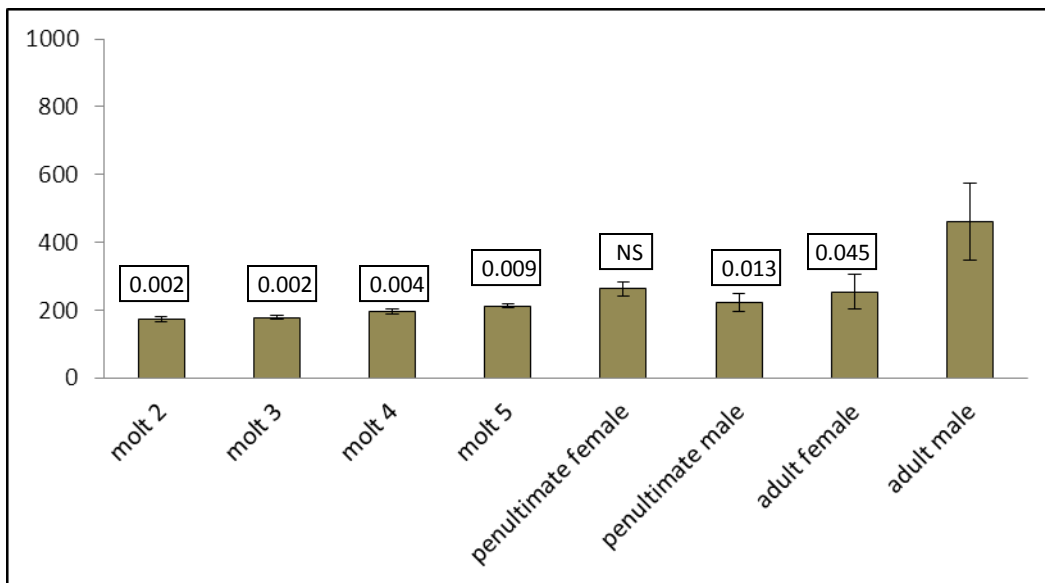
**Figure 3.1** Images taken of *A. diadematus* immature life stages under white light (left panel) and 340 nm (right panel). Molt stages are (a) 2, (b) 3, (c) 4, and (d) 5. Fluorescence images were taken without blocking filter. Bar = 1 mm. All right-panel images were set to gamma of .55 for ease of viewing



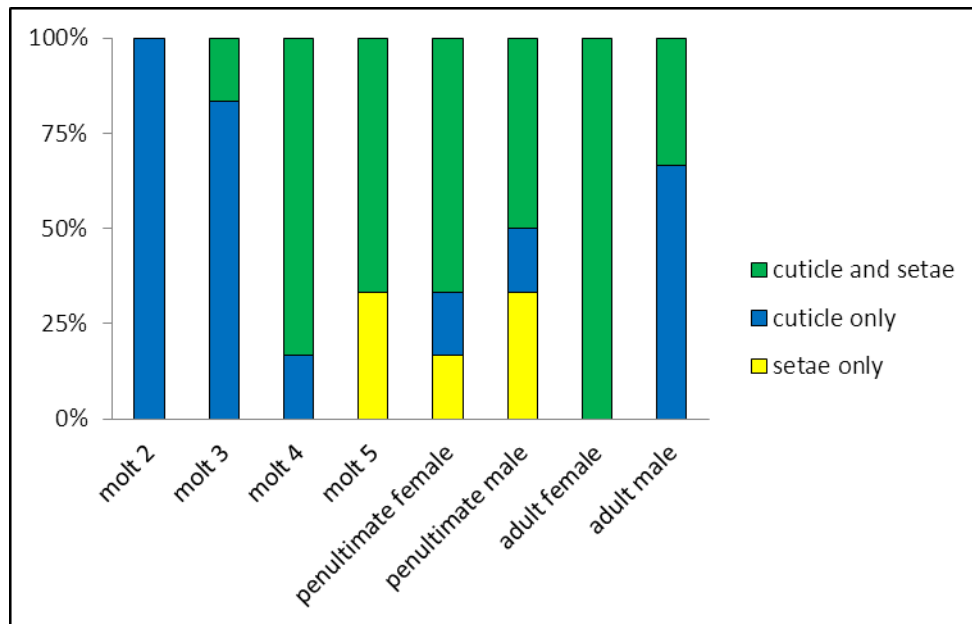
**Figure 3.2** Images taken of penultimate and adult *A. diadematus* under white light (left panel) and 340 nm (right panel). Molt stages are (e) penultimate female, (b) penultimate male, and (c) adult female, (d) adult male. Fluorescence images were taken without blocking filter. Bar = 1 mm. All right-panel images were set to gamma of .55 for ease of viewing.



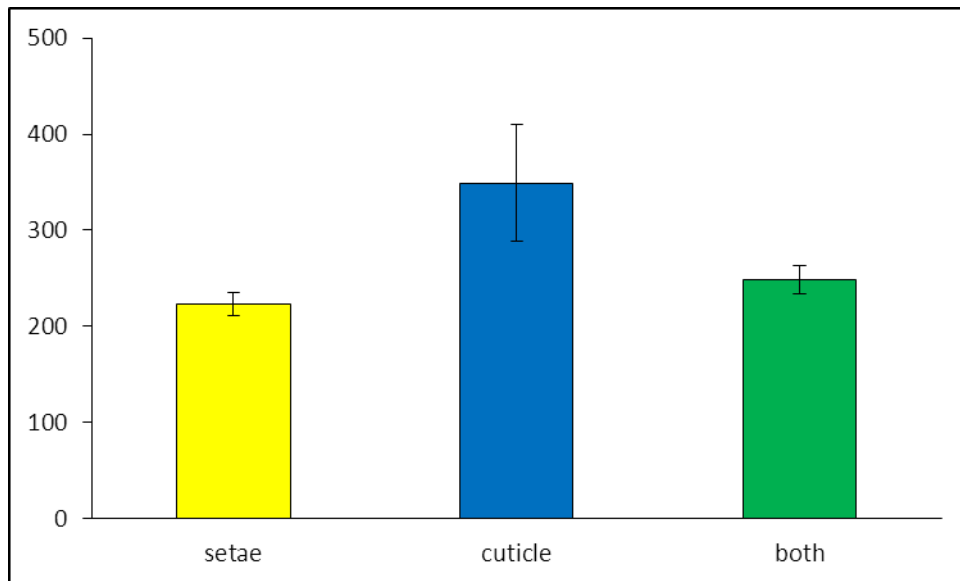
**Figure 3.3.** Average pixel intensity of abdomen for different sexes and life stages of *A. diadematus*. All images were taken at 340 nm without the blocking filter in place. Adult males show significantly brighter fluorescence from than all other life stages. All life stages are significantly different from adult males with a p value  $<<0.001$  (Tukey's HSD).



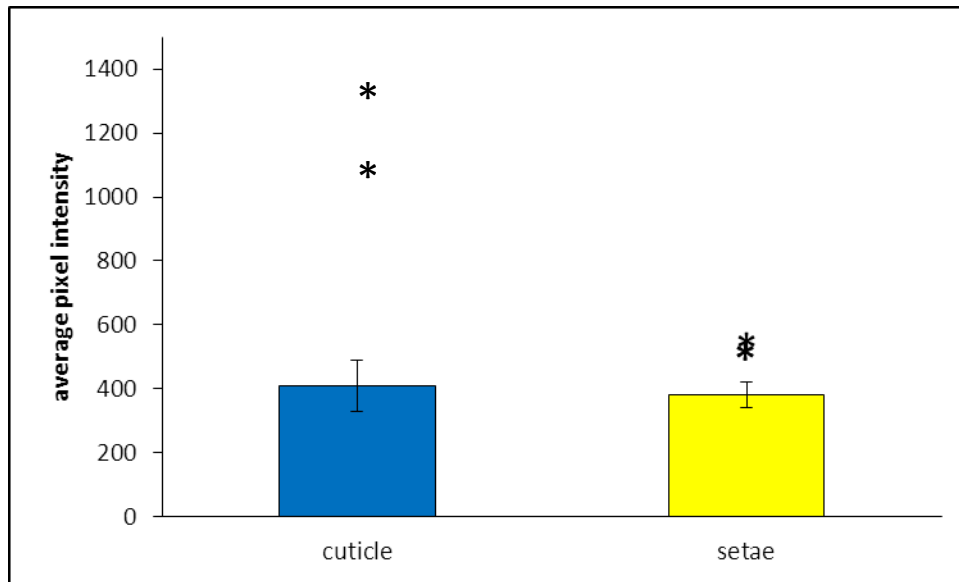
**Figure 3.4.** Average pixel intensity of the cephalothorax for different sexes and life stages of *A. diadematus*. All images were taken at 340 nm without the blocking filter in place. Adult males are significantly different from all other life stages except penultimate females. P value given above each bar (Tukey's HSD).



**Figure 3.5.** Distribution of fluorescent setae and cuticle across sexes and life stages in *A. diadematus*.



**Figure 3.6.** Overall fluorescence brightness in the abdomen in *A. diadematus* individuals with only fluorescent setae, only fluorescent cuticle, or both setae and cuticle. All life stages and sexes were combined for this analysis. None of the groups are significantly different from each other (Kruskal-Wallis test).



**Figure 3.7.** Average pixel intensity of fluorescent cuticle and setae patches. Representative individuals were taken from each life stage that had either fluorescent setae or cuticle. The groups are not significantly different (Wilcoxon Signed Rank Test). Readings from adult males are given by (\*).



## CHAPTER 4: Chapter 4 Survey of Fluorescence in Thomisidae Family

### INTRODUCTION

Spiders vary in both ecology and fluorescence intensity at all taxonomic levels (Andrews, Reed, and Masta 2007). Variation in fluorescence could be attributable to differences in ecology. A broad-scale comparison is an important component to understanding the implications of externally-expressed fluorescence in these animals. This chapter will discuss a family-wide comparison of fluorescence in the Thomisidae family.

Thomisids represent a diverse and speciose group, with 150 genera and over 1400 species within the family (Dondale and Redner 1978). Members of the Thomisidae family all have a “crab-like” scuttling walk, and no thomisids use webs to capture prey. Aside from these commonalities, there exists a great deal of diversity within the thomisids.

Thomisids vary in regards to ecology. Some species sit exposed to the sun on flowers. From these vantage points they can ambush pollinating insects. Others hunt prey in such varied places as leaf litter, dark crevices, or on tree bark (Dondale and Redner 1978). Some thomisids even specialize at hunting within tropical pitcher plants (Chua and Lim 2012).

Coloration in spiders often serves important ecological functions, be it for sexual signaling, camouflage, or prey attraction (Oxford and Gillespie 1998; M Thery and Casas 2009). Coloration is also important for thomisids. *M. vatia* is probably the best-studied thomisid from a coloration standpoint, as it has a remarkable ability to reversibly change color to match its surroundings (Morse 2007; T. C. Insausti and Casas 2009; T. Insausti and Casas 2008). Not all thomisids are able to change color, but coloration undoubtedly plays an important role in the lifestyle of these ambush predators.

Sexual dimorphism in size as well as color varies within the thomisids. Size dimorphism is common. Male thomisids tend to be smaller and longer-legged than females. Color dimorphism, however, is less common in Thomisidae than in other families of spiders, presumably because of their poor vision and lack of visual sexual displays (Oxford and Gillespie 1998; Dondale and Redner 1978). In thomisids which display color dimorphism, females are often larger, paler, and more uniformly colored than the males.

The overall purpose of this study was to determine whether coloration is influenced by fluorescence. We approached this question by evaluating fluorescence in a variety of thomisid species to determine whether fluorescence intensity is associated with ecological characteristics and coloration. In addition, we wanted to compare fluorescence intensity in males and females, and evaluate whether ecological differences between the sexes may be causing differences in fluorescence.

## MATERIALS AND METHODS

### **Specimen Collections**

To examine fluorescence expression in this group, thomisids were collected mostly from the Pacific Northwest region of the United States. Because species diversity was relatively high with a low sample size, it was difficult to compare individual species. Instead, we differentiated the thomisids into two groups based on coloration and ecology.

We divided our sample group into “white” (*Mecaphesa* and *Misumenoides*) and “dark” thomisids (*Bassaniana*, *Ozyptila*, and *Xysticus*). In general, the “white” thomisids tend to be exposed flower predators, whereas the “dark” thomisids spend more time in the leaf litter or in crevices in bark (Dondale and Redner 1978).

We sought to collect thomisids in different ecological niches, encompassing those that capture prey primarily on flowers, versus those that capture prey on the ground. We collected all specimens in the spring, summer, and fall of 2007-2011. A list of collection locations is given in table 4.1. Some specimens were collected as egg sacs or juveniles and reared to adulthood in the lab. Adult specimens were immediately frozen at -80 °C as described in chapter 1 methods. All specimens were identified to genus level. When possible, specimens were identified to species. See table 4.2 for a list of specimens.

**Imaging**

All specimens were thawed and imaged as described in chapter 1 methods.

Additionally, spectrometer measurements were taken as previously described.

Spectrometer measurements were averaged for each group of thomisids based on sex and taxonomy (“white” genera versus “dark” genera).

**Male-Female Fluorescence Comparisons**

Because we are primarily interested in whether fluorescence plays a role in coloration, we categorized our thomisids based on whether they were light or dark colored, as we found in preliminary studies that most taxa that have a dark coloration do not fluoresce very much (chapter ref). Thomisids from the PNW can be difficult to identify, requiring dissection, clearing, and drawing of genitalia to determine the species identity. Additionally, thomisid taxonomy is in a state of revision, with some groups still requiring further work to delimit species boundaries. It became clear during our study that the taxa in the PNW have not been adequately described. Hence, categorizing our taxa by their coloration and/ or ecology helped us avoid potential taxonomic errors.

In order to perform male-female fluorescence comparisons across thomisids, We compared pixel intensity (as a proxy for fluorescence brightness) in all white female thomisids to all white male thomisids, and all dark female thomisids to all dark male thomisids.

### **White-Dark Fluorescence Comparisons**

To ascertain whether ecology (sitting exposed in the sun or on the ground) affected fluorescence, we compared fluorescence brightness between all white thomisids and all dark thomisids, regardless of sex. For the first set of analyses we used a conservative approach, whereby thomisids were put into light and dark groups based on collection data. This placed some of the “dark” thomisids into the light group, as a few were found on sun-exposed flowers. Additionally, some specimens were omitted entirely as no collection information was known. In a second less-conservative approach, we divided spiders based solely on taxonomy, with the “white” genera considered synonymous with light-exposed, and the dark genera as the ground-dwellers.

## **RESULTS**

### **Reflectance Measurements**

The abdomens of adult white females had the brightest average reflectance compared to the abdomens of other spiders, especially at lower wavelengths (Figure 4.1). White males had lower reflectance than white females, but slightly brighter reflectance than all dark thomisids. Male and female dark thomisids had similar levels of reflectance.

### **Male-Female Fluorescence Comparisons**

Data for the abdomen intensity was normally distributed for all sexes ( $p > 0.050$ )

White males and females did not differ significantly in average pixel intensity for their

abdomens (t test) (Figure 4.2). The cephalothorax data were normally distributed for the white, but not dark thomisids. There were no significant differences between sexes for either coloration cephalothoraxes (t test for white, Wilcoxon Signed Rank Test for dark) (Figure 4.3). The right leg data was normally distributed for the dark, but not the white thomisids. There were no significant differences between sexes for dark Thomisids (t test), however male and female white Thomisid leg intensity did differ significantly ( $p = 0.020$ ) (Figure 4.4).

### **White-Dark Fluorescence Comparisons**

When male and female samples were pooled together and differentiated based on their known ecological characteristics, white thomisids were significantly brighter than dark thomisids in abdomen (t-test  $p < 0.001$ ), cephalothorax, (Wilcoxon Signed Rank Test  $p < .001$ ), and right leg (Wilcoxon Signed Rank Test  $p = 0.015$ ) (Figure 4.5). Data were normally distributed for abdomen, but not for cephalothorax or leg. In a less-conservative analysis in which spiders were divided based strictly on taxonomy, abdomen data were normally distributed, but not cephalothorax or leg data. Abdomens, (t-test  $p = 0.005$ ), cephalothoraxes (Wilcoxon Signed Rank Test  $p = 0.05$ ), and right legs (Wilcoxon Signed Rank Test  $p = 0.033$ ) differed significantly between white and dark thomisids (Figure 4.6).

## DISCUSSION

### **Fluorescence Differences between Sexes**

Within both color groups, males and females possessed similar levels of fluorescence brightness (Figure 4.3, 4.4). If camouflage is important to the ecology of thomisids, and fluorescence brightness contributes to camouflage, we thus might expect to see similar levels of fluorescence brightness if males and females had similar ecological characteristics. In spiders, a major aspect of their ecology consists of behaviors associated with hunting and feeding. Thus, we might expect males and females to have similar feeding habits.

Ecological information is scarce for thomisids, but in many spiders, including some thomisids, males and females are known to have dissimilar feeding behaviors (Elgar 1991; Morse 2007). Upon reaching the adult molt stage, females typically begin consuming large amounts of prey to provide for developing eggs. Males, on the other hand, often take an opposite approach and either stop eating, or eat very little so that they may spend their time searching for females (Foelix 2010). The similarity between males and females in regards to externally-expressed fluorescence is thus probably not due to similar feeding ecologies.

We might also expect to see similarities in fluorescence brightness between the sexes if predation pressures were similar. Even if males do not feed, they may court females in the same places that females feed, necessitating similar camouflage to avoid

predation. Thus, predation seems a more likely driving force maintaining similar levels of fluorescence between the sexes.

Another possible function for externally-expressed fluorescence is UV radiation protection. Fluorophores convert wavelengths from a short (high-energy) wavelength to a longer wavelength. Spider fluorophores excite in the potentially-dangerous ultraviolet and emit in the visible range, further suggesting a UV-protective role. This hypothesis has been proposed for fluorescence in corals as well (Salih et al. 2000). Thus, if fluorophores are photoprotective and males and females have the same exposure to sun, we might expect fluorescence brightness to be the same.

It seems possible that fluorophores could be photoprotective. However, the fluorophores that are creating the externally-visible fluorescence excite in the UVA range. These near ultraviolet wavelengths are less dangerous than shorter UVB wavelengths (Gallagher and Lee 2006). It is somewhat counterintuitive that spiders would have fluorophores to protect against the less-dangerous wavelengths but be unprotected from more dangerous ones. However, there is the possibility that other fluorophores are present that provide such a photoprotective function but we are unable to capture them with our imaging equipment.

### **White-Dark Fluorescence Differences**

If fluorescence were important for camouflage in thomisids, we would expect to see greater fluorescence intensity in spiders that inhabit environments exposed to sun



and on bright-colored backgrounds. Conversely, we would expect to see dimmer fluorescence in spiders that inhabit dark habitats and dark backgrounds.

When specimens were divided into white and dark, white thomisids had brighter fluorescence than dark thomisids (Figure 4.6, 4.7). This pattern coincides with the ecology of these two groups: white thomisids ambush prey from light-colored substrates, such as flowers, whereas dark thomisids inhabit niches with darker backgrounds, such as soil and leaf litter. The reflectance data also coincide with the fluorescence data. Dark thomisids had low reflectance overall (darker overall pigmentation), whereas white thomisids had brighter reflectance, especially in the ultraviolet (lighter coloration). Finally, fluorophores could also be serving a photoprotective role in the white thomisids, which are regularly exposed to the sun's damaging rays.

In habitats favored by dark thomisids, bright fluorescence would not be apparent even if there were fluorophores sequestered at the surface of the animal. We might then expect dark thomisids to possess the capability for bright external fluorescence if fluorophores were not costly to produce

Fluorescence in scorpions is a likely example of this situation. Scorpions possess fluorophores within the cuticle that cause the animal to fluoresce brightly under ultraviolet light. There is some debate over whether the fluorescence has an ecological function (Kloock, Kubli, and Reynolds 2010). However, it because scorpions are

nocturnal predators and therefore not typically exposed to the ultraviolet wavelengths present in the sun, ultraviolet excited fluorescence may simply be a by-product of some metabolic process.

Dark thomisids, by contrast, do not possess bright fluorescence even when exposed to the appropriate excitation wavelengths. One reason for this could be that dark thomisids may not need to fluoresce in their typical dark habitat. Fluorophores could have either been covered up or removed from visible areas due to metabolic expense and/or the need for other pigments.

Externally-visible fluorescence may instead (or additionally) have a direct cost for dark thomisids. Although dark thomisids tend to inhabit dark environments, they may be inadvertently exposed to the sun, for example, if a predator is searching through bark or leaf litter for prey. If the dark thomisids fluoresced brightly when thus exposed, they would be readily visible against the dark background.

#### CAVEATS

Some methodological problems arose in this study due to low sample size and identification of species. Each species was not analyzed independently for fluorescence, or on the basis of sex. Instead, it was necessary to average all members of one sex across several genera. This could have underestimated differences between sexes

For the white thomisids, it was impossible to determine species identification in the field. This created unevenness in the sample which could be improved with increased sample sizes. Also, different species of thomisids have distinct but overlapping breeding seasons. Re-visiting any given sampling site multiple times during the same year would capture more of this temporal diversity. Increased sampling would also benefit the dark thomisids sample, as sample sizes were generally low.

Another issue with low sample sizes for individual species is the possibility of color polymorphism within a species. This phenomenon is not well documented in thomisids, but in at least one species of *Xysticus*, individuals vary in color based on their hunting substrate (Bonte and Maelfait 2004). It would be useful to have a larger sample size of thomisids in order to address whether fluorescence (and reflectance) varies within, as well as between, different species.

## CONCLUSIONS

Fluorescence does not vary between sexes in either the light or the dark thomisids, but there are differences between the two groups when males and females are combined. This is likely due to differences in ecology between the white and dark thomisids. Camouflage has been previously shown to play an important role in thomisid ecology (Chittka 2001; Théry and Casas 2002; Morse 2007), so we might expect that the thomisids involved in the fluorescence study also utilize camouflage. If this were the case, we also might expect that fluorescence in spiders exposed to the sun on light-

colored substrates (such as flowers) to exhibit more fluorescence than those inhabiting darker habitats.

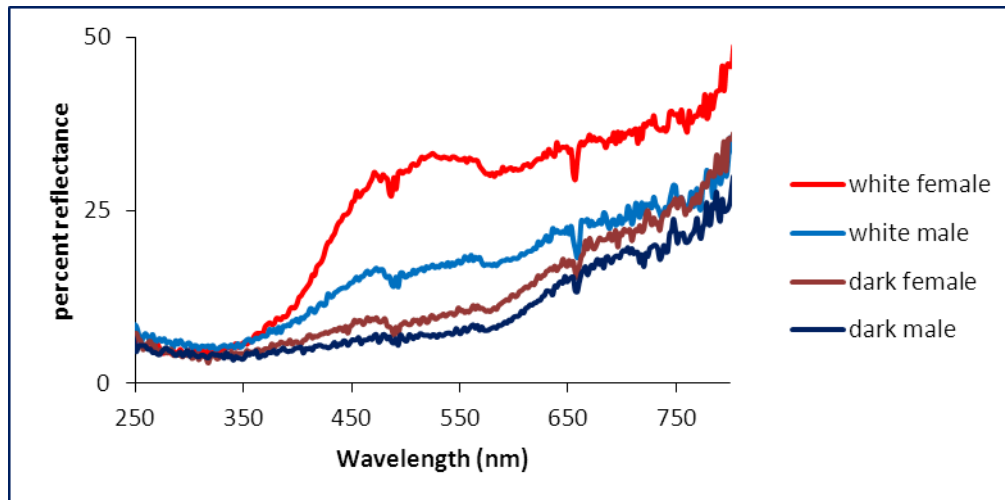
## TABLES AND FIGURES

**Table 4.1.** Collection Counties for Thomisid specimens

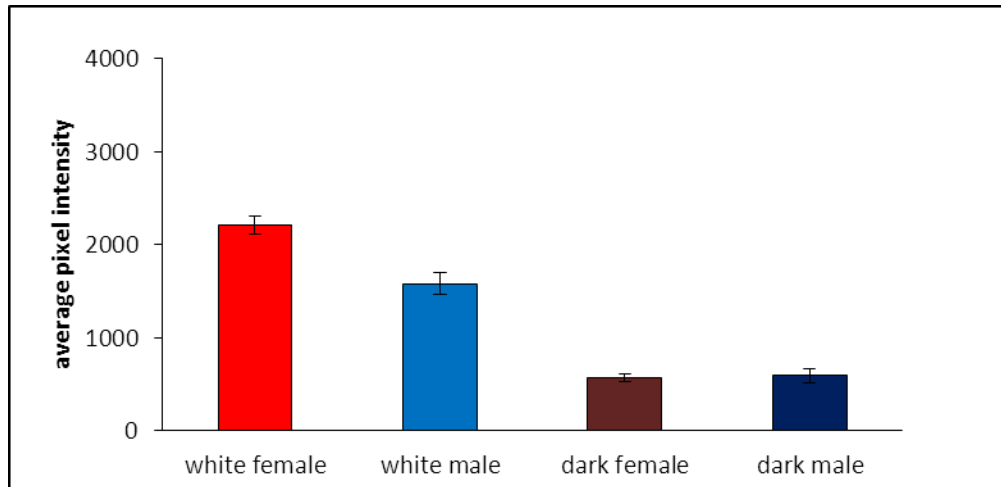
<b>Idaho</b>	Latah
<b>Michigan</b>	Oakland
<b>Oregon</b>	Clackamas
	Coos
	Curry
	Douglas
	Harney
	Hood River
	Jefferson
	Lane
	Lincoln
	Multnomah
	Tillamook
	Washington
<b>Washington</b>	Klickitat
	Skamania

**Table 4.2.** Specimen list including species identification when known

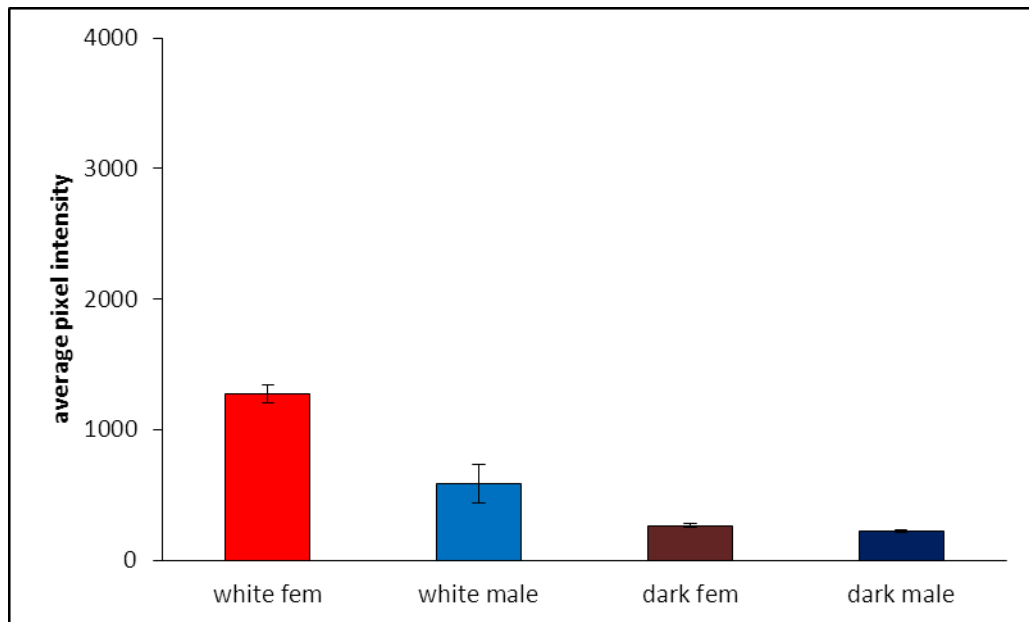
	Sex	Genus	Species	Number
White Thomisids (n = 15)	Female (n = 9)	<i>Mecaphesa</i>	<i>asperatus</i>	6
		<i>Mecaphesa</i>	<i>sirrensis</i>	1
		<i>Mecaphesa</i>	unknown	1
		<i>Misumenoides</i>	unknown	1
	Male (n = 6)	<i>Mecaphesa</i>	<i>carletonicus</i>	4
		<i>Mecaphesa</i>	<i>celer</i>	1
		<i>Mecaphesa</i>	unknown	1
Dark Thomisids (n = 13)	Female (n = 7)	<i>Bassaniana</i>	<i>utahensis</i>	1
		<i>Ozyptila</i>	<i>sp</i>	1
		<i>Xysticus</i>	<i>benefactor</i>	1
		<i>Xysticus</i>	<i>cuncator</i>	1
		<i>Xysticus</i>	<i>gosiutus</i>	1
		<i>Xysticus</i>	<i>locuples</i>	1
		<i>Xysticus</i>	unknown	1
	Male (n = 6)	<i>Xysticus</i>	<i>locuples</i>	2
		<i>Xysticus</i>	unknown	4



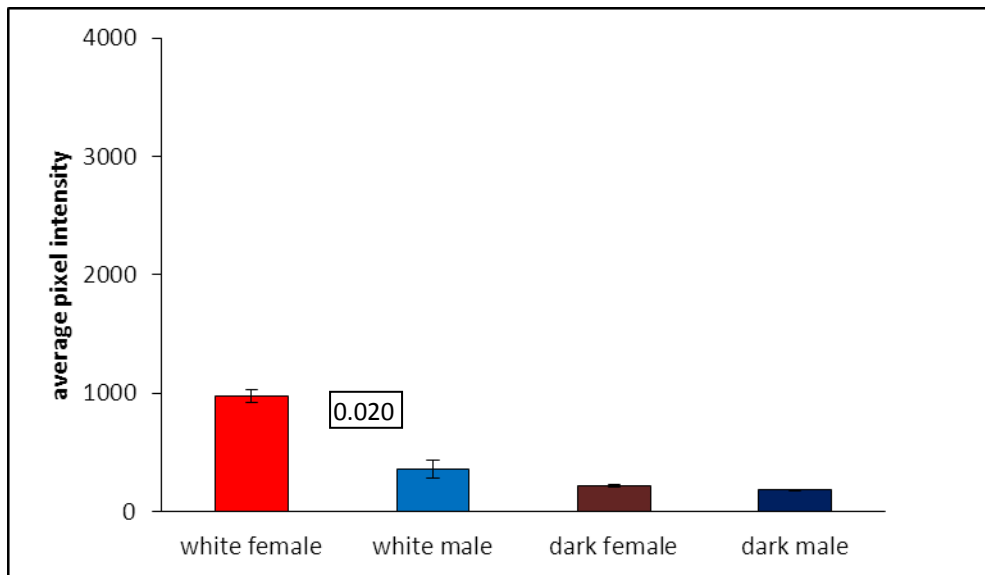
**Figure 4.1.** Average reflectance curves for white female, white male, dark female, and dark male Thomisids.



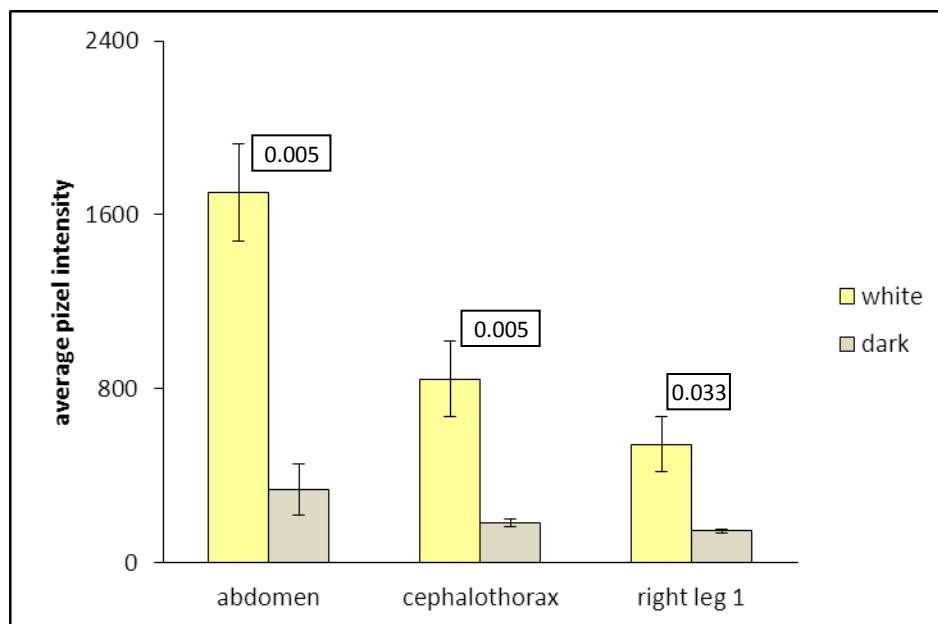
**Figure 4.2.** Differences in average fluorescence in abdomens between males and females of white and darker-pigmented Thomisids. Males and females do not differ from one another in either white or dark Thomisids. All images were taken at 340 nm without the blocking filter.



**Figure 4.3.** Differences in average fluorescence in cephalothoraxes between males and females of white and darker-pigmented Thomisids. Males and females do not differ from one another in either white or dark Thomisids. All images were taken at 340 nm without the blocking filter.

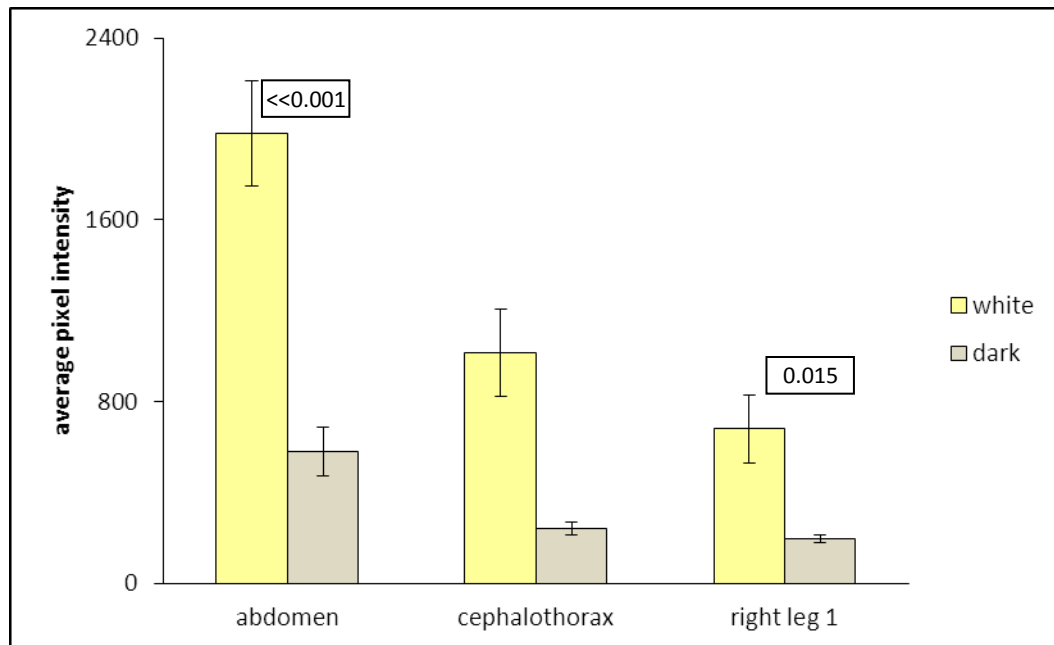


**Figure 4.4.** Differences in average fluorescence in right leg 1 between males and females of white and darker-pigmented Thomisids. Males and females do not differ from one another in dark Thomisids, but white males and females differ significantly (Wilcoxon Signed Rank Test, p value above bars). All images were taken at 340 nm without the blocking filter.



**Figure 4.5** Average fluorescence intensity between white and dark Thomisids (distinction based on ecological data). P values (t-test for abdomen, Wilcoxon Signed Rank for others) given above each pair of bars. All images were taken at 340 nm without the blocking filter.





**Figure 4.6.** Average fluorescence intensity between white and dark Thomisids (distinction based on taxonomy). P values (t-test for abdomen, Wilcoxon Signed Rank Test for others) given above each pair of bars. All images were taken at 340 nm without the blocking filter.

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